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ORIGINAL ARTICLE

Improvement of criteria for refractory cytopenia with multilineage dysplasia according to the WHO classification based on prognostic significance of morphological features in patients with refractory anemia according to the FAB classification

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In the criteria of refractory cytopenia with multilineage dysplasia (RCMD) according to the WHO (World Health Organization) classification, the frequency threshold concerning dysplasia of each lineage was defined as 10%. To predict overall survival (OS) and leukemia-free survival (LFS) for patients with refractory anemia (RA) according to the French-American-British (FAB) classification, we investigated prognostic factors based on the morphological features of 100 Japanese and 87 German FAB-RA patients, excluding 5q-syndrome. In the univariate analysis of all patients, pseudo-Pelger–Huet anomalies ⩾10% (Pelger+), micromegakaryocytes ⩾10% (mMgk+), dysgranulopoiesis (dys G) ⩾10% and dysmegakaryopoiesis (dys Mgk) ⩾40% were unfavorable prognostic factors for OS and LFS (OS; $P<0.001$, LFS; $P<0.001$). The prognostic effects of the morphological features were similar in both Japanese and German patients. However, dys Mgk ⩾10% was not correlated with OS and LFS. In the multivariate analysis, mMgk+ and dys Mgk⩾40% were adverse prognostic factors for OS for all patients, and dys G ⩾10% and dys Mgk⩾40% were adverse prognostic factors for LFS for all patients. On the basis of the present analysis, we propose the following modified morphological criteria for RCMD. Modified RCMD should be defined as FAB-RA, excluding 5q-syndrome with dys G ⩾10%, dys Mgk⩾40% or mMgk+.
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Introduction

Myelodysplastic syndromes (MDSs) are acquired clonal stem cell disorders characterized by ineffective hematopoiesis with myelodysplasia,[1] and are associated with a high risk of progression to acute leukemias.[2] MDSs are very heterogeneous in terms of their morphology, clinical features and survival.[3] Refractory anemia (RA) according to the French-American-British (FAB) classification is generally classified as a low-risk group.[4] The International Prognostic Scoring System (IPSS) was reported to be useful for assessing prognosis in MDS patients according to the FAB classification.[5] According to the WHO (World Health Organization) classification,[6] most FAB-RA patients are re-classified into refractory cytopenia with multilineage dysplasia (RCMD) or WHO-RA. It was reported that RCMD patients showed a more unfavorable prognosis than WHO-RA patients.[7–9] The criteria for RCMD include a uniform threshold of 10% for dysplasia in each lineage. However, the impact of this threshold on prognosis has not been fully assessed. Concerning the individual forms of dysplasia, we have previously reported that pseudo-Pelger–Huet anomalies (Pelger) and micromegakaryocytes (mMgk) were significantly correlated with overall survival (OS) and leukemia-free survival (LFS) in FAB-RA patients.[10,11] Here, we report the impact of the threshold for dysplasia in each lineage and the individual dysplasias on the prognosis of FAB-RA patients.

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Patients and methods

Patients

A total of 200 patients (Japan, 100 cases; Germany, 100 cases) with a diagnosis of primary FAB-RA were selected randomly. Patients who had previously been treated with antineoplastic drugs or ionizing radiation were excluded from the analysis. Japanese patients were diagnosed at the Saitama Medical University Hospital, Nagasaki University Hospital or affiliated hospitals in Japan between April 1976 and January 2002. German patients were diagnosed at the Department of Hematology, Oncology and Clinical Immunology of the Heinrich-Heine University in Germany between January 1973 and December 2002. Thirteen FAB-RA patients with isolated del(5q) (5q-syndrome; all were German patients) were excluded from the analysis. This retrospective analysis was performed in 100 Japanese and 87 German FAB-RA patients. Age, sex and cytogenetic findings of the patients at the diagnosis are summarized in Table 1.

Morphological study

Microscopical examinations were performed using standard methods (bone marrow (BM) Wright-Giemsa (WG) or May-Giemsa (MG), Prussian blue and periodic acid-Schiff stained films and peripheral blood (PB) WG or MG stained films). PB and BM differential counts were performed on 100 and 500 cells, respectively. Evaluations of the BM cellularity and number

Table 1 Results of morphological analysis and univariate analysis of OS and LFS in patients with FAB-RA, excluding 5q-syndrome

Variable	*No. of Patients*	*Percentile of OS* (months)			*Percentile of LFS* (months)			
		75%	*50%*	*P-value*	*90%*	*75%*	*50%*	*P-value*
All patients (n = 187)								
Age (years)								
Older than 60	96	21	59	<0.001	23	104	NR	0.053
60 or younger	91	68	202		53	NR	NR	
Sex								
Male	103	23	102	0.230	22	NR	NR	0.311
Female	84	44	NR		51	NR	NR	
Neutrophils								
Dys G								
⩾10%	41	20	33	<0.001	10	25	52	<0.001
<10%	122	54	175		104	NR	NR	
Pelger								
Positive*	26	16	29	<0.001	13	25	36	<0.001
Negative	153	31	158		52	NR	NR	
Dys G/Pelger								
Dys G⩾10% with Pelger positive*	26	16	29	0.114	13	25	36	0.455
Dys G⩾10% without Pelger positive*	15	20	52		10	28	NR	
Megakaryocytes								
Number of Mgk								
Normo/increased	154	23	109	0.083	25	NR	NR	0.046
Decreased	29	88	NR		NR	NR	NR	
Dys Mgk								
⩾10%	129	23	98	0.254	23	104	NR	0.101
<10%	25	23	176		NR	NR	NR	
Dys Mgk								
⩾40%	75	20	42	<0.001	14	36	NR	<0.001
<40%	79	88	217		NR	NR	NR	
mMgk								
Positive**	25	12	23	<0.001	13	25	51	<0.001
Negative	158	44	158		38	NR	NR	
Dys Mgk/mMgk								
Dys Mgk ⩾40% with mMgk positive**	24	12	23	<0.001	13	22	51	0.034
Dys Mgk ⩾40% without mMgk positive**	51	26	76		20	38	NR	
Chomosome (IPSS)								
Good	129	52	158	<0.001	74	NR	NR	<0.001
Int	35	20	NR		14	NR	NR	
Poor	23	7	27		4	22	31	
Japanese patients (n = 100)								
Age (years)								
Older than 60	42	20	44	<0.001	14	51	NR	0.005
60 or younger	58	56	157		NR	NR	NR	
Sex								
Male	53	29	176	0.802	25	NR	NR	0.702
Female	47	52	175		51	NR	NR	
Neutrophils								
Dys G								
⩾10%	17	22	29	<0.001	11	25	38	<0.001
<10%	79	88	176		104	NR	NR	
Pelger								
Positive*	12	27	31	0.003	22	25	38	0.010
Negative	87	52	176		74	NR	NR	
Dys G/Pelger								
Dys G⩾10% with Pelger positive*	12	27	31	0.724	22	25	38	0.481
Dys G⩾10% without Pelger positive*	5	5	20		4	4	NR	

Table 1 *Continued*

Variable	*No. of Patients*	*Percentile of OS (months)*			*Percentile of LFS (months)*			
		75%	*50%*	*P-value*	*90%*	*75%*	*50%*	*P-value*
Megakaryocytes								
Number of Mgk								
Normo/increased	81	31	175	0.411	37	NR	NR	0.234
Decreased	19	88	NR		NR	NR	NR	
Dys Mgk								
⩾10%	69	29	157	0.439	25	NR	NR	Not available
<10%	12	59	176		NR	NR	NR	
Dys Mgk								
⩾40%	38	23	52	0.001	22	38	NR	0.003
<40%	43	176	217		NR	NR	NR	
mMgk								
Positive**	12	22	23	<0.001	14	25	51	<0.001
Negative	88	62	176		74	NR	NR	
Dys Mgk/mMgk								
Dys Mgk ⩾40% with mMgk positive**	12	22	23	<0.001	14	25	51	0.019
Dys Mgk ⩾40% without mMgk positive**	26	42	106		37	NR	NR	
Chomosome (IPSS)								
Good	76	62	175	0.076	104	NR	NR	<0.001
Int	15	19	NR		NR	NR	NR	
Poor	9	29	38		4	25	37	
German patients ($n=87$)								
Age, y								
Older than 60	54	23	108	0.921	28	NR	NR	0.712
60 or younger	33	16	68		13	NR	NR	
Sex								
Male	50	20	54	0.137	20	NR	NR	0.308
Female	37	29	158		36	NR	NR	
Neutrophils								
Dys G								
⩾10%	24	16	36	0.040	10	20	52	<0.001
<10%	43	26	136		53	NR	NR	
Pelger								
Positive*	14	12	20	0.012	5	13	31	<0.001
Negative	66	23	68		52	NR	NR	
Dys G/Pelger								
Dys G ⩾10% with Pelger positive*	14	12	20	0.072	5	13	31	0.177
Dys G ⩾10% without Pelger positive*	10	43	65		10	52	NR	
Megakaryocytes								
Number of Mgk								
Normal/increased	73	16	54	0.159	23	53	NR	Not available
Decreased	10	44	108		NR	NR	NR	
Dys Mgk								
⩾10%	60	16	52	0.412	20	52	NR	0.419
<10%	13	22	NR		NR	NR	NR	
Dys Mgk								
⩾40%	37	12	29	0.001	13	28	53	0.001
<40%	36	65	NR		NR	NR	NR	
mMgk								
Positive**	13	10	16	<0.001	13	31	31	0.044
Negative	70	26	136		28	NR	NR	
Dys Mgk/mMgk								
Dys Mgk ⩾40% with mMgk positive**	12	9	16	0.019	5	13	31	0.489
Dys Mgk ⩾40% without mMgk positive**	25	20	43		20	28	53	
Chromosome (IPSS)								
Good	53	44	136	<0.001	52	NR	NR	<0.001
Int	20	26	65		13	NR	NR	
Poor	14	5	9		5	5	31	

Abbreviations: FAB, French-American-British; IPSS, International Prognostic Scoring System; LFS, leukemia-free survival; OS, overall survival; RA, refractory anemia.
Pelger positive*: the presence of 10% or more Pelger among 200 mature neutrophils.
mMgk positive**: the presence of 10% or more mMgk among 25 or more megakaryocytes.

of megakaryocytes were performed using the specimens of BM trephine biopsy and/or clot section.

We held two meetings on BM morphology at the Heinrich-Heine University, as reported previously.[12] At the first joint review, we mainly discussed the evaluation of dysplasia and diagnosis using the training slides. After the first joint review, the Japanese and German groups evaluated the detailed morphological analysis separately in each country. After this separate review, the second joint review meeting for morphological consensus was performed. The observers were blinded to the clinical and laboratory data, including cytogenetics, until finishing this joint review meeting for morphological consensus.

In the present study, we limited dysplasias to only dysplasias described in the WHO classification[6] as follows. Dysplasias of the nucleus in erythroid lineage cells were defined as budding, bridging, internuclear, karyorrhexis, multinuclearity or megaloblastoid change. Dysplasias of the cytoplasm in erythroid lineage cells were defined as ringed sideroblasts, vacuolization or PAS positivity (diffuse or granular). Concerning granulocytes, dysplasias were defined as small size, nuclear hypo-segmented mature neutrophils, hypersegmentation, hypogranularity or pseudo-Chediak-Higashi granules. Dysplasias of megakaryocytes were defined as micromegagakaryocytes, non-lobulated nuclei or multiple widely separated nuclei. A morphological study was performed in detail. A minimum of 25 megakaryocytes, 200 erythroblasts and 200 neutrophils in BM were examined in each patient. The cutoff levels for dyserythropoiesis (dys E) and dysgranulopoiesis (dys G) were defined as 10% according to the WHO classification. Dysmegakaryopoiesis (dys Mgk) was evaluated with two cutoff levels, 10% according to the WHO classification or 40% according to data previously reported from the German group.[7,13] Patients with decreased megakaryocytes were excluded from the evaluation of dys Mgk. Two distinct dysplastic changes, Pelger and mMgk, were also evaluated. We defined hypo-segmented mature neutrophils with strikingly clumpy chromatin as 'Pelger', and mono- or binucleated megakaryocytes with a size equal to or smaller than promyelocytes as 'mMgk'. Positivity for Pelger (Pelger+) was defined as the presence of 10% or more Pelger among 200 mature neutrophils. Positivity for mMgk (mMgk+) was defined as the presence of 10% or more mMgk among 25 or more megakaryocytes. Patients with decreased megakaryocytes were judged to be negative for mMgk (mMgk−). The final morphological evaluation was based on the consensus among the Japanese and German groups by joint review.

Cytogenetics

Cytogenetic analysis was performed with a trypsin-Giemsa banding technique on BM cells from aspirates. Ordinarily, 20–30 metaphases were examined. Cytogenetic aberrations were grouped according to the IPSS publication.

Statistical analysis

Patients were followed from the date of diagnosis until June 2004 for the Japanese and July 2003 for the German patients. Prognosis was evaluated by OS and LFS. OS was measured from the date of diagnosis until death owing to any cause, until the date of stem cell transplantation or until the last patient contact. LFS was measured from the date of diagnosis until the date of diagnosis of acute leukemia. Univariate analysis of sex, age category, each morphological parameter and cytogenetic subgroups according to IPSS on prognosis was evaluated with cumulative probabilities using the Kaplan–Meier method and compared using a log-rank test. Multivariate analysis was performed with several significant parameters from univariate analysis. The interaction between parameters was also examined. The effects of parameters were evaluated as hazard ratios and their 95% confidence intervals. Continuous data were compared using the nonparametric Mann–Whitney test, and proportions were compared using the χ^2 test. A two-sided *P*-value of <0.05 was considered to be statistically significant. All statistical analyses were performed with the use of StatView (version 5.0, SAS Institute, Cary, NC, USA) or SAS software (version 8.2, SAS Institute).

Results

Morphological analysis

In the 187 cases reviewed, we evaluated suitable marrow preparations for the detailed assessments of myelodysplasia. The results of morphological analysis are summarized in Table 1. All patients showed dys E$\geqslant$10%. Some marrow preparations could not be examined in detail. Especially concerning the frequency of dys G, 24 cases could not be evaluated, because the observation of granules of neutrophils was difficult owing to the poor staining condition of the films. Most of the patients with mMgk+ had dys Mgk$\geqslant$40%. Of the patients with dys Mgk$<$40%, only one patient had mMgk+. However, even with this patient it was judged that the frequency of dys Mgk was 36%.

Univariate analysis of the effects of each parameter on OS and LFS

Follow-up periods ranged from 1 to 292 months (median 43 months). During the follow-up period, 79 patients died and 24 patients transformed to acute leukemia. Japanese FAB-RA patients aged 60 years or less had a more favorable prognosis than German FAB-RA patients aged 60 years or less in OS ($P=0.001$). Table 1 shows the univariate analysis of the effects of each parameter on OS and LFS. In the analysis of all 187 patients, Pelger+, mMgk+, dys G $\geqslant$10% and dys Mgk$\geqslant$40% were significant adverse prognostic factors for OS and LFS. In a separate analysis for each country, there was no prognostic difference regarding Pelger+, mMgk+, dys G $\geqslant$10% or dys Mgk $\geqslant$40% between the Japanese and German patients. When cases with dys G $\geqslant$10% were divided into dys G $\geqslant$10% with or without Pelger+, the prognosis of dys G $\geqslant$10% with Pelger+ was not significantly different from that of dys G $\geqslant$10% without Pelger+ on OS and LFS in all patients and in the patients of each separate country. When cases with dys Mgk $\geqslant$40% were divided into dys Mgk $\geqslant$40% with or without mMgk+, the effect on the prognosis of dys Mgk $\geqslant$40% with mMgk+ was greater than that of dys Mgk $\geqslant$40% without mMgk+ on OS and LFS in all patients. In a separate analysis for each country, the prognosis of patients showing dys Mgk $\geqslant$40% with mMgk+ was worse than that of patients with dys Mgk $\geqslant$40% without mMgk+ regarding OS in patients of both countries and LFS in Japanese patients. Cytogenetic subgroups according to IPSS significantly affected OS and LFS in all patients. In a separate analysis for each country, they significantly affected OS in German patients and LFS in patients of both countries. Age $>$60 years significantly affected OS in all patients. In a separate analysis for each country, age $>$60 years significantly affected OS and LFS in only Japanese patients.

Table 2 Multivariate Cox hazard analysis of parameters for overall and leukemia-free survival in patients with FAB-RA, excluding 5q-syndrome

Characteristic	*OS*			*LFS*		
	Model A HR (95% CI)	*Model B HR (95%CI)*	*Model C HR (95% CI)*	*Model A HR (95% CI)*	*Model B HR (95% CI)*	*Model C HR (95% CI)*
All patients	$n=136$	$n=136$	$n=136$	$n=136$	$n=136$	$n=136$
Country, German (vs Japan)	1.1 (0.6–2.0)	1.3 (0.8–2.3)	1.1 (0.6–2.0)	1.1 (0.4–3.0)	1.2 (0.5–3.1)	0.9 (0.4–2.3)
Age, older than 60 years (vs 60 years or younger)	1.7 (0.9–3.1)	1.8 (1.0–3.1)*	2.1 (1.1–3.7)*	1.6 (0.6–4.0)	1.6 (0.6–3.9)	3.0 (1.1–8.3)*
Sex, male (vs female)	1.6 (0.9–2.7)	1.3 (0.7–2.3)	1.1 (0.6–2.0)	2.4 (0.9–6.2)	2.3 (0.8–6.2)	2.0 (0.7–5.4)
Dys G$\geqslant$10% (vs<10%)	2.1 (1.1–3.8)*	1.6 (0.7–3.7)	1.5 (0.6–3.4)	5.3 (2.0–14)*	5.4 (1.5–19)*	6.3 (1.7–23)*
Pelger, positive (vs negative)		1.2 (0.5–3.2)	1.2 (0.5–3.1)		0.8 (0.2–3.0)	0.7 (0.2–2.6)
Dys Mgk$\geqslant$40% (vs<40%)	2.7 (1.5–5.0)*	1.9 (1.0–3.8)*	1.9 (0.9–3.7)	5.6 (1.6–20)*	5.0 (1.3–18)*	3.9 (1.0–15)*
mMgk, positive (vs negative)		3.6 (1.8–7.4)*	3.1 (1.4–6.7)*		2.0 (0.7–6.0)	0.9 (0.2–3.2)
Chomosome (IPSS), low			1			1
Chomosome (IPSS), intermediate			2.4 (1.2–4.9)*			3.1 (0.9–11)
Chomosome (IPSS), poor			2.5 (1.1–5.7)*			10.5 (2.7–40)*
Japanese patients	$n=78$	$n=78$	$n=78$	$n=78$	$n=78$	$n=78$
Age, older than 60 years (vs 60 years or younger)	6.3 (2.7–15)*	5.9 (2.4–14)*	6.1 (2.5–15)*	5.4 (1.5–20)*	5.0 (1.3–20)*	7.3 (1.7–30)*
Sex, male (vs female)	1.1 (0.5–2.3)	1.1 (0.5–2.4)	1.0 (0.5–2.1)	1.5 (0.4–5.0)	1.7 (0.5–6.6)	1.9 (0.4–7.6)
Dys G$\geqslant$10% (vs<10%)	3.5 (1.4–8.8)*	4.9 (1.2–19)*	4.6 (1.1–19)*	5.1 (1.2–23)*	11.1 (1.5–82)*	9.6 (1.1–83)*
Pelger, positive (vs negative)		0.5 (0.1–2.1)	0.7 (0.1–3.0)		0.2 (0.1–2.1)	0.2 (0.1–2.3)
Dys Mgk$\geqslant$40% (vs<40%)	2.1 (0.9–4.5)	1.8 (0.8–4.2)	2.1 (0.9–5.2)	4.9 (1.0–25)*	4.2 (0.8–24)	4.4 (0.7–28)
mMgk, positive (vs negative)		1.7 (0.6–5.3)	1.2 (0.4–4.1)		2.1 (0.4–11)	1.0 (0.1–7.6)
Chomosome (IPSS), low			1			1
Chomosome (IPSS), intermediate			2.8 (0.9–8.6)			2.7 (0.2–31)
Chomosome (IPSS), poor			1.4 (0.4–4.8)			6.7 (1.2–37)*
German patients	$n=58$	$n=58$	$n=58$	$n=58$	$n=58$	$n=58$
Age, older than 60 years (vs 60 years or younger)	0.5 (0.2–1.1)	0.7 (0.3–2.0)	1.1 (0.4–3.0)	0.5 (0.1–1.7)	0.3 (0.1–1.6)	3.1 (0.2–53)
Sex, male (vs female)	1.4 (0.6–3.5)	1.3 (0.5–3.2)	1.1 (0.4–2.7)	3.4 (0.8–14)	3.8 (0.8–18)	2.1 (0.4–10)
Dys G$\geqslant$10% (vs<10%)	1.9 (0.8–4.5)	1.7 (0.5–5.7)	1.6 (0.5–5.1)	12.8 (2.3–72)*	18.0 (1.8–181)*	28.5 (2.1–380)*
Pelger, positive (vs negative)		1.1 (0.3–4.1)	0.9 (0.2–3.4)		0.8 (0.1–5.0)	0.5 (0.1–3.7)
Dys Mgk$\geqslant$40% (vs<40%)	3.6 (1.3–9.9)*	2.5 (0.8–8.0)	2.0 (0.6–6.2)	7.3 (0.9–60)	10.1 (1.0–105)*	4.4 (0.4–48)
mMgk, positive (vs negative)		2.4 (0.8–7.4)	2.5 (0.8–7.6)		0.5 (0.1–3.4)	0.4 (0.1–3.0)
Chomosome (IPSS), low			1			1
Chomosome (IPSS), intermediate			2.9 (1.0–8.8)*			4.7 (0.7–34)
Chomosome (IPSS), poor			4.4 (1.2–16)*			59.6 (0.9–4165)

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; IPSS, International Prognostic Scoring System; OS, overall survival; LFS, leukemia-free survival.
Model A included country category, age, sex, dichotomized dysgranulopoiesis and dysmegakaryopoiesis.
Model B included country category, age, sex, dichotomized dysgranulopoiesis, Pelger, dichotomized dysmegakaryopoiesis and micromegakaryocytes.
Model C included country category, age, sex, dichotomized dysgranulopoiesis, Pelger, dichotomized dysmegakaryopoiesis, micromegakaryocytes and cytogenetic findings.
*Statistically significant hazard ratio.

Multivariate analysis of the effects of each parameter on OS and LFS

As a next step, we performed a multivariate analysis based on our results obtained in univariate analysis. Table 2 shows the multivariate analysis of the effects of each parameter on OS and LFS. We analyzed parameters in all patients and in patients of each country separately. Model A included the country, age category, sex, dys G category and dys Mgk category. Model B included the country, age category, sex, dys G category, Pelger, dys Mgk category and mMgk. Model C included parameters of model B and cytogenetic findings. In model A, dys G $\geqslant$10% and dys Mgk$\geqslant$40% were significantly associated with an adverse prognosis regarding OS and LFS in all patients. In Japanese patients, dys G $\geqslant$10% was a significant adverse prognostic factor for OS and LFS, and Mgk $\geqslant$40% was a significant adverse prognostic factor for LFS. In German patients, dys G $\geqslant$10% was a significant adverse prognostic factor for LFS, and Mgk $\geqslant$40% was a significant adverse prognostic factor for OS. In model B, dys Mgk $\geqslant$40% and mMgk+ were significantly associated with an adverse prognosis regarding OS, and dys G $\geqslant$10% and dys Mgk $\geqslant$40% were significant adverse prognostic factors for LFS in all patients. In Japanese patients, dys G $\geqslant$10% was a significant adverse prognostic factor for OS and LFS. In German patients, dys G $\geqslant$10% and dys Mgk $\geqslant$40% were significant adverse prognostic factors for LFS. In model C, mMgk+ and cytogenetic subgroups were significantly associated with an adverse prognosis regarding OS, and dys G $\geqslant$10%, dys Mgk $\geqslant$40% and cytogenetic subgroups were significant adverse prognostic factors for LFS in all patients. In Japanese patients, dys G $\geqslant$10% was a significant adverse prognostic factor for OS, and dys G $\geqslant$10% and cytogenetic subgroups were significant adverse prognostic factors for LFS. In German patients,

cytogenetic subgroups were a significant adverse prognostic factor for OS, and dys G ⩾10% was a significant adverse prognostic factor for LFS. Age >60 years was a significant adverse prognostic factor for OS and LFS of all models in Japanese patients. In contrast, age >60 years was not a significant adverse prognostic factor in German patients.

Proposal for morphological criteria for RCMD

Regarding OS, RCMD patients who were diagnosed using a uniform threshold of 10% for dys G and dys Mgk according to the original WHO classification did not show a worse prognosis than WHO-RA patients ($P=0.111$) (Figure 1). This finding indicates that the morphological criteria for RCMD of the original WHO classification may be insufficient for assessing the prognosis. Dys Mgk ⩾10% was not correlated with OS and LFS. However, the frequency of dys Mgk was correlated with prognosis in FAB-RA patients, excluding 5q-syndrome. Patients with dys Mgk ⩾70% or dys Mgk of 40–70% showed a more unfavorable prognosis than patients with dys Mgk of 10–40% or dys Mgk<10% (OS, $P<0.001$; LFS, $P<0.001$). Patients with dys Mgk ⩾70% had a more unfavorable prognosis than patients with dys Mgk of 40–70% (OS, $P=0.003$; LFS, $P=0.114$). However, there was no prognostic difference between the patients with dys Mgk of 10–40% and patients with dys Mgk<10% (OS, $P=0.277$; LFS, $P=0.881$). (Figure 2) Most of the patients with mMgk+ had dys Mgk⩾40%. Of the patients with dys Mgk<40%, only one patient had mMgk+. The prognostic effect of mMgk+ might relate to the prognostic difference between patients with dys Mgk ⩾40% and patients with dys Mgk<40%. To clarify this point, we compared the OS and LFS between patients showing dys Mgk⩾40% without mMgk+ (dys Mgk⩾40%/mMgk−) and patients with dys Mgk<40%. However, patients with dys Mgk⩾40%/mMgk− had a more unfavorable prognosis than patients with dys Mgk<40% (median survival: dys Mgk⩾40%/mMgk−, 76 months; dys Mgk<40%, 217 months; $P=0.001$, 10%; LFS: dys Mgk⩾40%/mMgk−, 20 months; dys Mgk<40%, not reached; 25% LFS: dys Mgk⩾40%/mMgk−, 38 months; $P<0.001$). In addition, dys Mgk⩾40% and mMgk+ were independent adverse prognostic factors for OS in the model B of multivariate analysis.

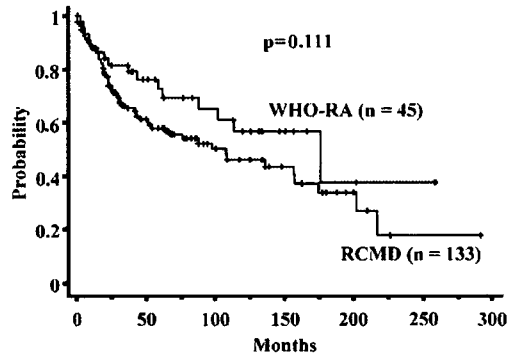

Figure 1 Cumulative overall survival and leukemia-free survival of WHO-RA and RCMD patients according to the original WHO classification. In overall survival, the RCMD patients, according to original WHO classification, did not show a more unfavorable prognosis than the original WHO-RA patients ($P=0.111$).

We attempted to modify the original WHO criteria for RCMD. Except for 5q-syndrome, the WHO classification for the MDS category does not include cytogenetic findings. Therefore, we excluded the cytogenetic findings from the parameters for RCMD. Based on uni- and multivariate analyses, we propose modified morphological criteria for RCMD, as shown in the following. FAB-RA patients, excluding 5q-syndrome, are re-classified into RCMD or WHO-RA. Category A is defined as dys E⩾10%. Categories B1, B2 and B3 are defined as dys G⩾10%, dys Mgk⩾40% and mMgk+, respectively. RCMD is diagnosed when category A and any other category B are present. WHO-RA is defined as FAB-RA other than RCMD. Of the 173 present patients who were suitable for a detailed assessment of dysplasia, our FAB patients, excluding 5q-syndrome, were re-classified into 89 modified WHO-RA and 84 modified RCMD patients according to our modified morphological criteria. Frequency of the 'poor risk karyotype' according to IPSS in the modified WHO-RA (5%) was lower than that in the modified RCMD (20%) ($P=0.002$). In contrast, the frequency of the 'good risk karyotype' in the modified WHO-RA (80%) was higher than that in modified RCMD (61%) ($P=0.006$). In the OS, modified RCMD patients were significantly more unfavorable than modified WHO-RA patients (Figure 3a). For patients aged 60 years or less, the OS of the modified RCMD patients was significantly more unfavorable than that of the modified WHO-RA patients. And, for those older than 60 years, the modified

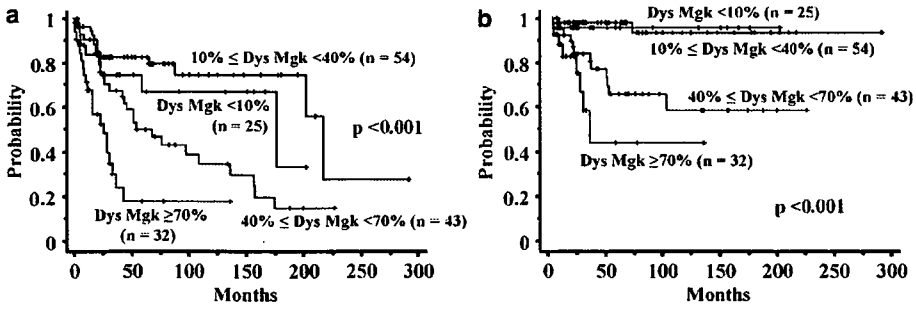

Figure 2 Cumulative overall and leukemia-free survival and frequency of dysmegakaryopoiesis in patients with FAB-RA, excluding 5q-syndrome. (**a**) Overall survival (OS). (**b**) Leukemia-free survival (LFS). (**a**) Patients with dys Mgk ⩾70% or dys Mgk of 40–70% showed a more unfavorable OS than patients with dys Mgk of 10–40% or dys Mgk<10% ($P<0.001$). Patients with dys Mgk ⩾70% had a more unfavorable OS than patients with dys Mgk of 40–70% ($P=0.003$). There was no prognostic difference between patients with dys Mgk of 10–40% and patients with dys Mgk<10% ($P=0.277$). (**b**) Patients with dys Mgk ⩾70% or dys Mgk of 40–70% showed a more unfavorable LFS than patients with dys Mgk of 10–40% or dys Mgk<10% ($P<0.001$). The LFS of patients with dys Mgk ⩾70% tended to be worse than that of patients with dys Mgk of 40–70% ($P=0.114$). There was no prognostic difference between patients with dys Mgk of 10–40% and patients with dys Mgk<10% ($P=0.881$).

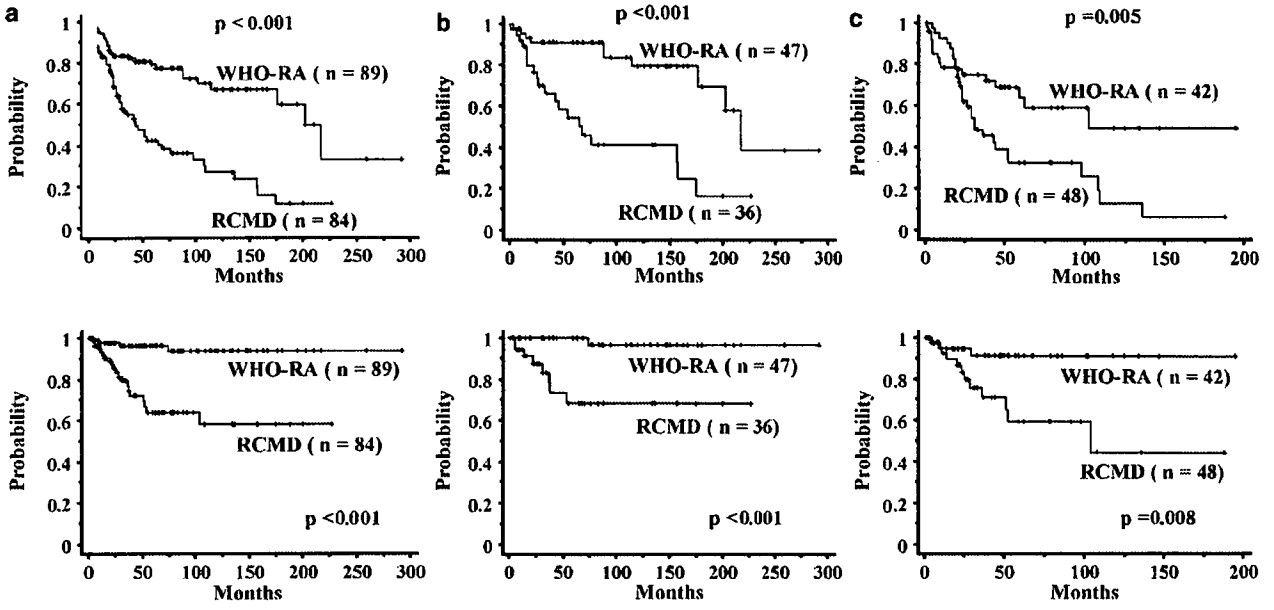

Figure 3 Cumulative overall survival and leukemia-free survival of WHO-RA and RCMD patients according to the modified WHO classification. (Top) Overall survival (OS). (Bottom) Leukemia-free survival (LFS). (**a**) Among all patients with FAB-RA, excluding 5q-syndrome, the modified RCMD patients had a more unfavorable prognosis than the modified WHO-RA patients (OS, $P<0.001$; LFS, $P<0.001$). (**b**) In patients aged 60 years or younger, the modified RCMD patients had a more unfavorable prognosis than the modified WHO-RA patients (OS, $P<0.001$; LFS, $P<.0.001$). (**c**) In patients aged older than 60 years, the modified RCMD patients had a more unfavorable prognosis than the modified WHO-RA patients (OS, $P=0.005$; LFS, $P=0.008$).

RCMD patients show a more unfavorable OS than the modified WHO-RA patients (Figure 3b-c). In the LFS, the modified RCMD patients were significantly more unfavorable than the modified WHO-RA patients (Figure 3a). For patients aged 60 years or less, the LFS of modified RCMD patients was significantly more unfavorable than that of the modified WHO-RA patients. For those older than 60 years, the modified RCMD patients show a more unfavorable LFS than the modified WHO-RA patients (Figure 3b-c).

Discussion

The patients in the present study were selected randomly, and the number of patients was smaller than our previous study.[12] However, the clinical features of Japanese patients were different from those of German patients as in the previous study. Japanese patients in present study were significantly younger than German patients (median age: Japan, 56 years; Germany, 62 years; $P=0.026$). Japanese patients had lower absolute neutrophil counts (median: Japan, 1.39×10^9/l; Germany, 1.82×10^9/l; $P=0.069$), lower hemoglobin concentrations (median: Japan, 8.2 g/dl; Germany, 10.3 g/dl; $P<0.001$), lower platelet counts (median: Japan, 34×10^9/l; Germany, 108×10^9/l; $P<0.001$), and a lower frequency of cytogenetic abnormalities (Japan, 27%; Germany, 64%; $P<0.001$) than German patients.

Previous reports from our Japanese and German MDS study group, as well as other investigators, confirmed that WHO-RA patients had a more favorable prognosis than RCMD patients.[7–9,12] In our previous report,[12] the concordance rate of morphological diagnosis according to the WHO classification between Japanese and German hematologists was 83.8%, and a significant concordance was achieved while using the WHO classification (κ 0.73, $P<0.001$). Therefore, we believe that the evaluation of the frequency of dysmyelopoiesis is comparable between Japanese and German hematologists. Moreover, the present final evaluations concerning dysplasia were reached by consensus among the Japanese and German groups by a joint review. We believe that the WHO classification based on morphological features is useful, at least, in our Japanese and German groups, if the morphological features reflect the prognosis. However, the previous study[12] was performed according to the criteria of a prior report from Germany.[7] The threshold of dys Mgk in this report was defined as 40%. In the criteria of RCMD according to the original WHO classification, the threshold of frequency for the degree of dysplasia in each lineage was defined as 10%. Still, the impact of this threshold (10%) in each lineage on prognosis has not been fully assessed. Nosslinger *et al.*[14] reported that WHO-RA patients did not show more favorable prognoses when compared to the RCMD patients. However, the threshold of dysplasia in their report was 50%. This demonstrates that the threshold of dysplasia for RCMD is still controversial. In the present study, all patients showed dys E$\geqslant$10%. Therefore, dys E$\geqslant$10% did not have a prognostic effect. Dys Mgk$\geqslant$10% was not an unfavorable prognostic factor for OS and LFS. On the other hand, dys G $\geqslant$10% and dys Mgk$\geqslant$40% were significant adverse prognostic factors correlated with OS and LFS. And, these threshold levels have similar prognostic effects between Japanese and German patients in uni- and multivariate analyses. We reported earlier that Pelger and mMgk were correlated with OS and LFS in Japanese patients.[10,11] However, when mature neutrophils had two lobes, the definition of 'Pelger' in this previous report was different from that in the present study. In this previous report, we defined mature neutrophils with the two lobes joined by a thin, hair-like bridge ('pince-nez type cells') as 'Pelger'. In contrast, we defined hypo-segmented mature neutrophils with

strikingly clumpy chromatin as 'Pelger' in the present study. Because of this difference of the definition, the frequency of Pelger in the present study was higher than that in this previous report. In the present study, Pelger+ and mMgk+ were significant adverse prognostic factors for OS and LFS. Again, these results were similar between the Japanese and German patients in uni- and multivariate analyses. The results of the present study support our previous results not only in Japanese patients, but also in German patients. Of note, the prognosis of dys G $\geqslant$10% with Pelger+ was not different from that of dys G $\geqslant$10% without Pelger+. In contrast, the prognosis of dys Mgk $\geqslant$40% with mMgk+ was worse than that of dys Mgk $\geqslant$40% without mMgk+.

We recently compared the clinical features of Japanese and German patients with FAB-RA and found some different prognostic factors, e.g. cytopenias according to IPSS publication were found to be useful for the assessment of prognosis in German FAB-RA patients, but not in Japanese FAB-RA patients.[12] In contrast, the prognostic relevance of the morphological features was similar in Japanese and German FAB-RA patients in the study presented here. However, in the multivariate analyses, there were slight differences between Japanese and German FAB-RA patients. For this reason, we speculate that the prognostic effects of the age category of Japanese patients may have an influence. In the present study, Japanese FAB-RA patients aged 60 years or less had a more favorable prognosis than German FAB-RA patients aged 60 years or less in OS ($P=0.001$) as in our previous study.[12] The degree of dysplasias was more severe in Japanese patients aged older than 60 years than those 60 years or younger. The frequency of RCMD according to our modified criteria was higher in Japanese patients aged older than 60 years than those 60 years or younger (48% vs 35%). Therefore, it seems that morphological features may not be significant independent prognostic factors due to the effects of age category in Japanese patients. In contrast, there were no differences in the frequency of RCMD according to our modified criteria between the German patients aged older than 60 years and those 60 years or younger (60% vs 62%). In addition, because the frequency of poor staining of the films was high in German patients, only a small number of cases could be judged in the morphological study ($n=58$) of German patients. It is expected that the significance as prognostic factors of morphological features becomes certain in multivariate analyses if we can examine more examples even in German patients. Concerning model C including cytogenetics, the number of patients with poor karyotype among 84 RCMD patients according to the modified definition was 17 (20%). In contrast, the number of patients with poor karyotype among 89 WHO-RA patients was only 4 (5%). We thought that the degree of dysplasias was related to the cytogenetic findings. Therefore, in model C, morphological features may not be significant independent prognostic factors.

In univariate analyses, dys G $\geqslant$10% was correlated with OS and LFS in all patients and in patients from each country. However, dys Mgk $\geqslant$10% was not correlated with OS and LFS in all patients or in patients from either country. In the present patients, RCMD patients diagnosed by using a uniform threshold of 10% for dys G and dys Mgk according to the original WHO classification did not show a worse prognosis than WHO-RA patients. We think that it may be necessary to revise the morphological definition of RCMD to improve the WHO classification. Therefore, we propose modified morphological criteria for RCMD.

This morphological analysis in MDS patients has several limitations. We held two meetings on BM morphology and made great efforts to achieve morphological consensus. However, the evaluation of dysplasias might be different among different observers. The number of evaluable cells in the megakaryocytic lineage is smaller than that in other lineages. Therefore, the concordance rate of frequency of dys Mgk among different observers might be lower than that of dys G or dys E. We think that the different morphological interpretation of megakaryocytes among different observers is one of the main causes of the disagreement in the diagnosis of WHO classification. For example, patients of FAB-RA in which three megakaryocytes among 25 megakaryocytes are judged to be dysplastic are classified as RCMD according to the original WHO classification. In contrast, patients of FAB-RA in which only two megakaryocytes among 25 megakaryocytes are judged to be dysplastic are classified as WHO-RA. Therefore, we think that this threshold (10%) of dys Mgk has problems not only for the assessing of the prognosis but also for the diagnosis of RCMD. It was reported that mMgk was specific dysplasias in MDS patients[15] and the concordance rates concerning mMgk was sufficient.[10] We think that the disagreement rate of morphological diagnosis might be decreased by using our modified criteria combining the frequency of dys Mgk and mMgk. The threshold (40%) of dys Mgk of our modified criteria is different from that (10%) of the original WHO criteria. The German group had already shown the usefulness of the WHO classification in several large-scale studies[7,13] using this threshold (40%). Therefore, we believe that the usefulness of this threshold shown in the present study is certain.

In conclusion, the present results showed that the degree of dysplasia in FAB-RA patients was related to OS and LFS, and the prognostic effect of dysplasia was similar between the Japanese and German FAB-RA patients. However, the thresholds of dysplasia influencing prognosis were different from the original threshold of the WHO classification. We propose to raise the threshold of dys Mgk in the criteria for RCMD from 10 to 40% and add mMgk+.

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ORIGINAL ARTICLE

Small number of HTLV-1-positive cells frequently remains during complete remission after allogeneic hematopoietic stem cell transplantation that are heterogeneous in origin among cases with adult T-cell leukemia/lymphoma

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can provide long-term remission for patients with adult T-cell leukemia/lymphoma (ATLL) caused by human retrovirus, human T-lymphocyte virus (HTLV-1). To understand how HTLV-1-positive cells including ATLL cells were suppressed by allo-HSCT, we examined HTLV-1 provirus load and residual ATLL cells in peripheral blood of transplant recipients using PCR-based tests. We found that the copy number of HTLV-1 genome, called provirus, became very small in number after allo-HSCT; however, in most cases, provirus did not disappear even among long-term survivors. Tumor-specific PCR tests demonstrated that most of HTLV-1-positive cells that remained long after transplantation were not primary ATLL cells but donor-derived HTLV-1-positive cells. We also found a case having very low amount of residual disease in peripheral blood even long after transplantation. There was only one recipient in whom we failed to show the presence of HTLV-1 genome and antibody against HTLV-1 even with an extensive search, which strongly suggested the elimination of HTLV-1 after allo-HSCT. These results demonstrated that after allo-HSCT the small amount of residual HTLV-1-positive cells were heterogeneous in origin and that long-term disease control for ATLL could be obtained without the complete elimination of HTLV-1.
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Keywords: ATLL; transplantation; MRD; HTLV-1

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell lymphoma caused by a retrovirus, human T-lymphocyte virus (HTLV-1), which randomly integrates into the genome of infected T cells.[1–3] The HTLV-1 genome in T cells, called provirus, has been utilized for the diagnosis of the disease caused by or the carrier state of HTLV-1. For example, Southern blot analysis of HTLV-1, when it demonstrates a monoclonal proliferation of cells infected with HTLV-1, provides the strongest evidence for the diagnosis of ATLL.[4] Southern blot analysis usually detects a monoclonal population composed of 3–5% of total cells, which is generally enough to diagnose ATLL. On the other hand, polymerase chain reaction (PCR)-based tests detect HTLV-1 genome with much higher sensitivity than Southern blot analysis, allowing us to monitor a small amount of HTLV-1 provirus load.[5,6]

The clinical course of ATLL widely differs by clinical subtypes (acute, lymphoma, chronic and smoldering). The prognoses of acute and lymphoma types are very poor when treated with conventional or even high-dose chemotherapy;[7,8] however, with allogeneic hematopoietic stem cell transplantation (allo-HSCT), a long-term clinical remission (CR) is achievable as reported from several groups including ours.[9–11] For example, among cases with acute ATLL, allo-HSCT reduced the volume of tumor cells in the peripheral blood to undetectable level when tested by morphological examination or Southern blot analysis, suggesting that the reduction of ATLL cells was less than 5% of WBC, as we reported previously.[11]

In this study, as an extension of our previous report, to understand how small the population of HTLV-1-positive cells would become after allo-HSCT and to test whether HTLV-1 could be eradicated, we investigated HTLV-1 provirus load and the minimum residual disease (MRD) in 22 cases of ATLL using PCR-based gene amplification. Since PCR for HTLV-1 provirus picked up not only ATLL cells, but also all cells infected with HTLV-1, including polyclonal non-ATLL cells, we introduced a specific PCR method to detect ATLL cells utilizing a unique integration site of HTLV-1 in each ATLL case.

We found that cells carrying HTLV-1 existed at the very low level in peripheral blood of long-term survivors after allo-HSCT. Most of them were donor-derived cells, but MRD was simultaneously present only in one case. We also experienced a single case in which anti-HTLV-1 antibodies became negative with no HLTV-1 genome amplified with PCR-based tests, suggesting the eradication of HTLV-1.

Patients and methods

Clinical features of patients with ATLL

The diagnosis and classification of ATLL was based on the criteria proposed by the Lymphoma Study Group of Japan.[12] Twenty-two patients with the diagnosis of acute or lymphoma type ATLL who received allo-HSCT in three hospitals in Nagasaki, an endemic area of HTLV-1 in Japan, between September 1997 and May 2004 were included in this study.

Table 1 summarizes the clinical characteristics of these patients. Median age of the patients was 48 years. In 21 of all 22 cases, donor-derived hematopoiesis was obtained (Table 2).

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Table 1 Characteristics of patients and transplantation

Case no.	*Age at HSCT /sex*	*Disease status at HSCT*	*Donor α HTLV-1 Ab*	*Donor*	*Source*	*Conditioning Regimen*
1	44/M	NC	–	Related	BM	TBI-VP16-CA
2	48/M	PD	–	Related	BM	TBI-VP16-CA
3	43/F	CR	–	Unrelated	BM	TBI-VP16-CA
4	51/M	PR	–	Related	BM	BU-CY2
5	30/F	PR	+	Related	BM	BU-CY3
6	54/F	PR	+	Related	BM	BU-CY2
7	44/F	PR	–	Unrelated	BM	TBI-CY
8	48/F	CR	+	Related	BM	BU-CY3
9	35/M	PD	–	Related	PB	BU-CY2
10	39/M	PD	–	Related	PB	TBI-CY
11	41/F	NC	–	Unrelated	BM	TBI-CY
12	48/M	PR	–	Related	PB	TBI-CY
13	46/M	PR	+	Related	BM	TBI-CY
14	50/F	PR	–	Unrelated	BM	TBI-CY
15	63/M	PD	+	Related	PB	FLU-BU-ATG (RIST)
16	53/M	CR	–	Related	PB	FLU+L-PAM (RIST)
17	55/M	NC	–	Related	PB	FLU-BU (RIST)
18	63/M	NC	–	Related	PB	FLU-CY (RIST)
19	48/F	PR	+	Related	BM	FLU+L-PAM (RIST)
20	53/M	CR	+	Related	BM	FLU-CY (RIST)
21	56/M	PD	+	Related	PB	FLU+L-PAM+TBI (RIST)
22	62/M	PR	–	Related	PB	FLU-BU (RIST)

Abbreviations: BM, bone marrow; BU, busulfan; CA, Cytarabine; CR, complete response; CY, cyclophosphamide; FLU, fludarabin; HSCT, hematopoietic stem cell transplantation; L-PAM, melphalan; NC, no change; PB, peripheral blood; PD, progressive disease; PR, partial response; RIST, reduced-intensity conditioning transplantation; TBI, total body irradiation; VP16, Etoposide.

Table 2 Results of transplantation

Case no.	*Engraftment*	*Relapse*	*aGVHD*	*cGVHD*	*Outcome*
1	+	Day 3074	I	—	Alive with ATLL (day 3094+)
2	+	—	IV	NE	Died of GVHD on day 123
3	+	Day 144	II	—	Died of ATLL on day 165
4	+	Day 169	I	—	Died of ATLL on day 237
5	+	—	0	—	Alive in CR (day 1756+)
6	+	Day 833	II	—	Alive in 2nd CR (2nd CR after local irradiation, day 1679+)
7	+	Day 262	0	Extensive	Died of ATLL on day 1310
8	+	—	0	Extensive	Alive in CR (day 1497+)
9	+	—	III	—	Died of infection on day 137
10	+	—	0	Limited	Alive in CR
11	+	Day 78	I	Extensive	Died of ATLL on day 218
12	+	—	III	—	Alive in CR (day 254+)
13	+	—	IV	—	Died of TRM on day 120
14	+	—	0	—	Died of cerebral haemorrhage on day 216
15	+	—	0	Extensive	Died of GVHD on day 167
16	+	—	II	Limited	Alive in CR (day 1138+)
17	+	—	I	Extensive	Alive in CR (day 1087+)
18	+	—	III	Extensive	Died of infection on day 370
19	+	—	II	—	Alive in CR (day419+)
20	–	—	NE	NE	Alive in CR with recipient-derived hematopoiesis (day 580)
21	+	NE	0	NE	Died of infection on day 41
22	+	Day 73	I	Extensive	Alive with ATLL (day 184+)

Abbreviation: aGVHD, acute GVHD; cGVHD, chronic GVHD; NE, not eligible.

Only one patient (case 21) did not achieve CR after allo-HSCT and seven patients experienced a relapse of ATLL. At the time of analysis, 11 patients were alive and nine of these patients remained in CR.

Quantitative measurement of HTLV-I provirus load in peripheral blood

Peripheral blood samples were collected from the patients after they gave a written informed consent. Genomic DNA was extracted from mononuclear cells (MNC) of peripheral blood using the QIAGEN DNA Midi Kit (QIAGEN, Hiden, Germany) and from paraffin-embedded sample using DEXPAT (TAKARA BIO INC, Shiga, Japan). Quantitative measurement of HTLV-1 provirus was performed with real-time quantitative PCR (RQ-PCR) using the LightCycler System and DNA Master Syber Green I (Roche diagnostics, Mannheim, Germany) as reported previously.[13] In brief, 30 ng of genomic DNA was used as a template and the copy number of HTLV-1 provirus was assessed by the ratio of the amount of tax region of HTLV-1 and that of beta globin gene (tax copies/MNC = 2 × copy number of tax/copy number of beta-globin gene). The mean value of two

experiments was shown as the copy number of HTLV-1 provirus load. Figure 1 shows the correlation between the ratios of the positive control plasmid containing tax region in the irrelevant plasmids and the results of RQ-PCR tests in a log-scale graph. A statistically significant correlation was found ($r=0.89$, $P<0.001$). This system could quantify one copy of the tax gene in 5000 cells.

Detection of primary ATLL cells with inverse PCR

To detect the residual ATLL cells, we performed an inverse PCR as reported by Takemoto *et al.*[14] that amplified the integration site of HTLV-1 in the genome of tumor cells whose sequence was then utilized to establish case-specific PCR primers that amplified a part of HTLV-1 (LTR) and the franking region. Each PCR in this study could at least detect one primary ATLL cell among 10000 normal cells. PCR condition and the DNA sequence of the primer sets in nine cases tested are available upon request.

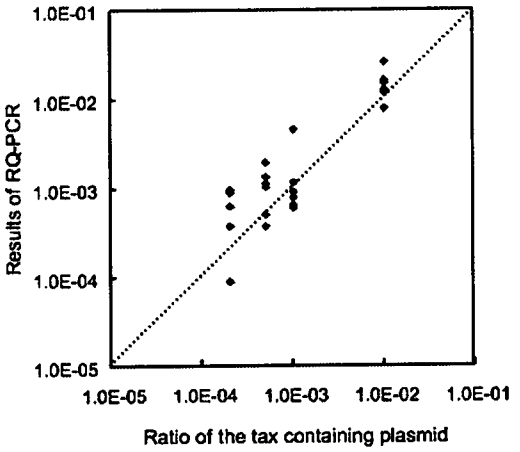

Figure 1 Correlation of the ratio of tax copy number between control plasmid and the quantification using RQ-PCR. Control plasmids containing the tax region of HTLV-1 were serially diluted with plasmids containing irrelevant sequence (beta-globin) and the ratio of target plasmid was quantified using the RQ-PCR method.

Colony formation and the expansion of HTLV-I-infected cells to test the origin of those cells

Previously, we established a method to clonally amplify HTLV-I-infected cells.[15] In brief, MNC in the peripheral blood were cultured in semisolid media containing 0.93% methylcellulose dissolved in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (FCS) and 200 ng/ml of recombinant human interleukin (rhIL)-2 (TECHNE Corp., Minneapolis, MN, USA). After three weeks of culture, each colony grown in the semisolid media was picked up individually and transferred to liquid culture (IMDM with 20% FCS and 20 ng/ml of rhIL-2) for clonal expansion. All cell culture was performed at 37°C with 5% CO_2. The origin of cells (donor or recipient) was assessed by means of sex mismatch (using Y chromosome specific SRY gene detection) or the difference of the number in short tandem repeat (STR method).

Results

Quantitative measurement of HTLV-I provirus after allo-HSCT

A total of 86 samples in 22 patients were collected; samples per patient were from 1 to 10 (median 3.5 samples) with median sampling time of 6 months from transplant (0.5 month to 8.3 years). The copy numbers of HTLV-1 provirus in each case are shown in Figure 2a and b. Most of the samples contained a low amount of HTLV-1 provirus, except for two conditions: (1)

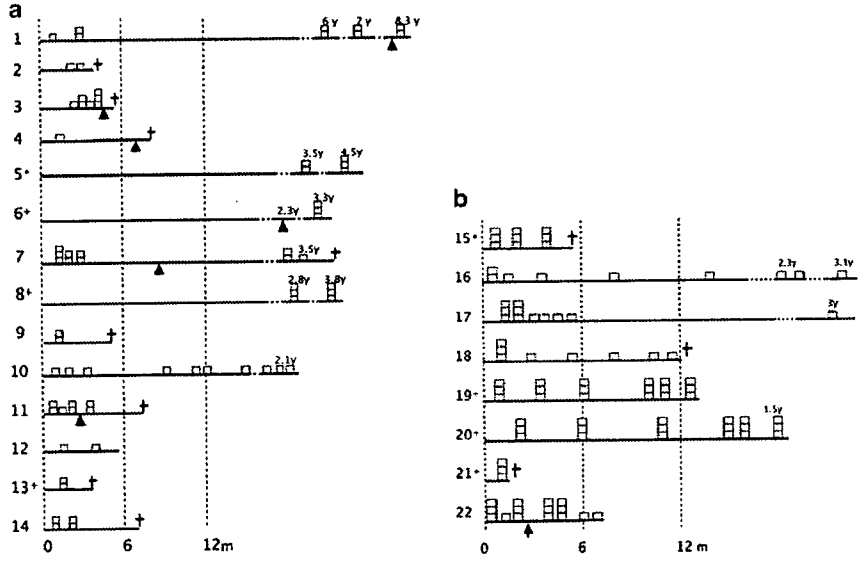

Figure 2 Quantification of HTLV-1 provirus load in the peripheral blood of recipients. Case number is on the left side of the figures. Case number with plus mark represents transplantation from a carrier donor. Copy number of provirus is shown as a gray or white box: three gray boxes represent virus load $\geq 10^{-2}$; two gray boxes, $10^{-2} \sim 10^{-3}$; one gray box, $10^{-3} \sim 10^{-5}$; white box, below detection level. Time after transplantation is described as month (m) or year (y). Cross mark represents death of the case and arrow indicates the time of relapse of ATLL. Cases treated with myeloablative conditioning are shown in (**a**) and those received RIST are in (**b**).

transplantation from a carrier donor and (2) right before (about 2 weeks) or after the clinical relapse of ATLL. In 22 samples transplanted from carrier donors, the provirus load was always 500 copies/10^5 cells or more despite the clinical disease status at sampling. The average copy number of HTLV-1 was significantly higher in patients transplanted from a carrier donor than from a noncarrier donor (mean value, 15000 and 760 copy/10^5 cells, respectively, $P<0.0001$).

Within 6 months from transplantation, the provirus load became undetectable at least once in eight out of 15 cases (case numbers 2, 3, 4, 10, 12, 16, 17 and 18). However, in all seven cases tested later, the copy number of HTLV-1 provirus became detectable again. At the time of the last follow-up, provirus load was below the detection level in only two cases (case numbers 16 and 17). The provirus load during the early period following transplantation was not related to the type of conditioning regimen, disease status before the transplantation or the duration of survival. There was no statistically significant association between provirus loads and the development of severe acute GVHD (data not shown). No specific pattern in the kinetics of virus load was noticed among long-term survivors or among patients that experienced relapse.

Analysis of MRD in the peripheral blood

As a low level of HTLV-1 provirus load was detected in the peripheral blood of most patients, we tested whether primary ATLL cells remained as MRD using specific PCR for primary ATLL cells, which amplified a unique franking genomic region of the HTLV-1 integration site in each case. In nine cases (cases 1, 5, 9, 10, 15, 18, 19, 21 and 22), 34 samples were analyzed with this method (Table 3 and Figure 3). Although the sensitivity of the inverse PCR varied from case to case, the amount of MRD that could be detected by this method was always below the provirus load quantified by RQ-PCR in every sample (data not shown).

Eighteen out of 19 samples collected after this period were negative for MRD regardless of the presence of HTLV-1 provirus. An exception was the sample taken at the time of relapse that took place 8.3 years after transplantation in case 1. CR was continuously maintained in this case and the peripheral blood samples at 6 and 7 years from transplantation were negative in the MRD test. A subcutaneous tumor, which developed at relapse, consisting mostly of CD4-positive cells, had the same integration site of HTLV-1 as primary ATLL cells, demonstrating that the primary ATLL cells had persisted for more than 8 years as MRD.

Analysis of the origin of cells carrying HTLV-1 provirus

Although most of the cells carrying provirus were HTLV-1-infected cells and were not derived from ATLL clones, these findings raised the question of whether these infected cells derived from recipients or donors. To answer this question, we cultured peripheral blood MNC in semisolid media in the presence of rhIL-2 to clonally expand cells infected with HTLV-1. Among 10 cases that maintained CR more than a year, samples were obtained from eight cases. In five out of eight cases, we could establish 30 cell lines (Table 4). Each cell line contained HTLV-1 provirus (data not shown).

In case 20, in which the graft was rejected after transplantation, all eight cell lines were derived from the recipient cells. Among other four cases, 22 out of 23 cell lines were found to originate from the donor cells including one cell line of case 1 that received transplantation from a noncarrier donor. In case 5, despite long-term CR (4.5 years) and complete donor chimerism in the peripheral blood, there was one cell line (one of seven cell lines) that derived from a recipient. By using the established cell line of recipient origin, we determined the franking genomic sequence of HTLV-1 integration site and set up the inverse-PCR. It was applied retrospectively to the genomic DNA extracted from a paraffin-embedded lymph node, which was a biopsy sample for the initial diagnosis in case 5. The lymph node

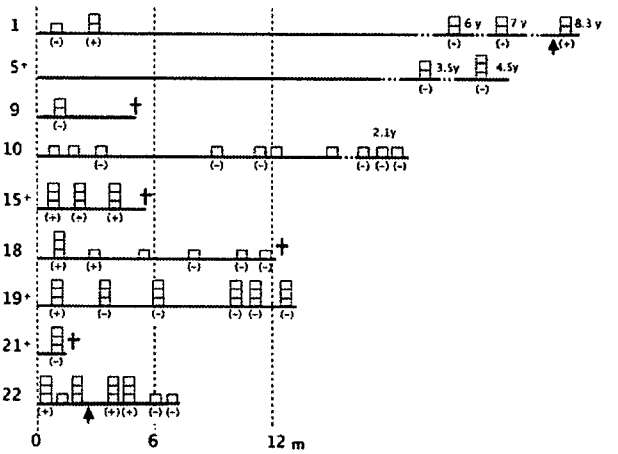

Figure 3 MRD of ATLL after transplantation. MRD of ATLL was assessed using case-specific inverse PCR method. Results of the inverse PCR are shown under the boxes that represent the copy number of provirus. Marks in this figure are the same as in Figure 2.

Table 3 DNA sequence of the franking region of HTLV-1 integration site

Case no.	*DNA sequence of the integration site*
1	AAATTTAGTACACAatatactatgacatataaagtatatgaggt...
5	AAATTTAGTACACAcagatctttccaggaaagataactttaaaa...
9	AAATTTAGTACACAtgcattaagttgaaagctggaaaaattaaa...
10	AAATTTAGTACACAaaaatgtacccaggatttgttttaatcagt...
15	AAATTTAGTACACAaggcataagccagatttacattataaatgc...
18	AAATTTAGTACACAaaatgtaaaaagcctcaagaaattgtaagc...
19	AAATTTAGTACACAgtttctaatcttattttgctgtgcaagctg...
21	AAATTTAGTACACAcatatgaactttaaagtagttttttccaat...
22	AAATTTAGTACACAggcacccagcctaaacccactgctacctga...

DNA sequence of the part of the 3′ region of HTLV-1 integration sites are shown.
Upper cases indicate the sequence of LTR.

Table 4 Origin of colony-forming cells in recipients

Case no.	*Anti-HTLV-1 Ab in the donor*	*Time (year) after HSCT at sampling*	*Number of IL-2-dependent cell lines*		
			Total	*Donor derived*	*Recipient derived*
1	–	7	1	1	0
5	+	4.5	7	6	1
8	+	3.8	5	5	0
10	–	2.1	0	—	—
16	–	2.3 and 3.1	0	—	—
18	–	1	0	—	—
19	+	1.1	9	9	0
20*	+	1.5	8	0	8

*Graft was rejected in case 20.

Table 5 Serial tests for anti HTLV-1 antibody and provirus in case 16

Time after transplantation	*3 weeks*	*1.5 months*	*0.5 year*	*1 year*	*1.6 years*	*2.3 years*	*3.1 years*
Anti-HTLV-1 antibody (PA assay)	NT	NT	x 16	UD	NT	UD	UD[a]
Proviral load	1.86×10^{-3}	UD	UD	UD	UD	UD	UD
Nested PCR test for pX region	NT	UD	UD	Positive	UD	UD	UD
PCR test for gag region	NT	NT	NT	NT	UD	UD	UD
PCR test for env region	NT	NT	NT	NT	UD	UD	UD
IL-2-dependent CFC	NT	NT	NT	NT	2 colonies	0	0

Abbreviations: CFC, colony-forming cell; NT, not tested; UD, undetectable.
[a]Undetectable with three different methods; Western blotting, particle agglutination and fluorescent antibody test.

sample had the same integration site of HTLV-1 as the cell line established 4.5 years after transplantation. Although two peripheral blood samples taken 4.5 years after transplantation were negative for this inverse-PCR, the colony-formation method could detect MRD in the same sample in case 5.

Negative results in the tests for HTLV-1 infection in case 16

In cases 16 and 17, at the time of the last follow-up, HTLV-1 provirus load was below the sensitivity of PCR (1 provirus/10^5 cells). However, the test for antibody against HTLV-1, which is widely used to demonstrate the infection with HTLV-1, was found to be negative only in case 16 (Table 5). Three different methods (Western blotting, particle agglutination and fluorescent antibody test) failed to demonstrate antibodies against HTLV-1 in this case. PCR tests for other parts apart from tax of HTLV-1, gag and env regions, were also negative. All extensive searches for HTLV-1 infection became negative 2.3 years after transplantation and remained negative 8 months later, 3.1 years from transplantation when this manuscript was written.

Discussion

In the present study, we measured HTLV-1 provirus load, detected MRD and determined the origin of HTLV-1 positive cells in the peripheral blood in 22 cases with ATLL treated with allo-HSCT. The HTLV-1 provirus load was reduced at least once to low levels (less than 1000 copies/10^5 cells) in most cases even among those who were transplanted in the status other than CR or those who received a reduced-intensity conditioning. These results showed a strong anti-ATLL effect of allo-HSCT in the short period after transplantation. The average dose of HTLV-1 provirus was significantly higher among cases transplanted from HTLV-1 carrier donors, suggesting the carryover of the virus positive cells from the donors. However, the level of provirus load after transplant did not always correlate to the final clinical outcome. Surprisingly, among most of the patients who survived more than 2 years, HTLV-1 provirus was detectable, although at a lower level, by PCR in their peripheral blood. Contrary to our results, Hishizawa *et al.*[16] using a quantitative PCR method similar to ours, reported the kinetics of HTLV-1 provirus load after allo-HSCT in five cases with ATLL, and they showed that HTLV-1 provirus load was undetectable in two cases in continuous CR. Major differences between their report and ours are the length of the follow-up period (1–15 and 1–84 months) and the number of patients (five and 22 cases). The longer observation periods and larger case number in our study might have facilitated the notice of the reappearance of HTLV-1-positive cells after allo-HSCT.

In contrast with the frequent positive results of provirus load, MRD of primary ATLL was rarely detectable after transplantation. In particular, after 6 months from transplantation, all samples of five cases tested during remission were negative for the MRD test despite the detectable level of provirus load, clearly demonstrating the presence of HTLV-1-positive cells other than ATLL in the peripheral blood of these patients.

HTLV-1-positive cells present in the recipients after allo-HSCT could be theoretically categorized into four groups: (1) MRD of primary ATLL cells, (2) non-ATLL cells of a recipient carrying HTLV-1 (e.g. T lymphocytes at the carrier state), (3) donor-derived cells infected with HTLV-1 in the host after transplant and (4) infused donor cells in the case of transplantation from a carrier of HTLV-1. Based on the results of colony-formation experiments, although the number of clones tested was not large, we demonstrated that there was difference in the origin of cells with HTLV-1 provirus. We found MRD in case 5 (as defined in group 1), donor-derived HTLV-1-positive cells in case 1 (group 3) and examples of group 4 in cases 5, 8 and 19. Non-ATLL cells of recipients were shown in case 20 (group 2). In some cases, we assumed that donor CD4-positive T cells were infected *de novo* with HTLV-1 in the recipient's body after transplantation as observed in case 1. Virus transmission into donor lymphocytes was described previously and our observation supported this report.[17]

In case 1, the MRD tests in the peripheral blood were negative in both samples taken at 6 and 7 years from transplantation; however, ATLL relapsed clinically as a subcutaneous tumor after 8 years of continuous CR. With the same integration sites of HTLV-1 in the primary and relapsed tumor cells, it was apparent that the primary ATLL cells remained somewhere in the body for more than 8 years after allo-HSCT and that negative tests for MRD in the peripheral blood did not necessarily indicate eradication of ATLL even long after transplantation.

On the other hand, in case 16, even with the extensive search for HTLV-1 provirus by PCR for various parts of HTLV-1 genome, we failed to demonstrate its presence in the peripheral blood. The antibody against HTLV-1 also became negative only in this case. So far, there has been no evidence to show the presence of HTLV-1 in this case for more than 8 months. There was a previous report of the eradication of HTLV-1 from a carrier who received allo-HSCT for pure red cell aplasia.[18] The tests for the virus performed in case 16 were almost the same as used in this report, suggesting that HTLV-1 was cleared off from the body after allo-HSCT in this case, indicating eradication of both ATLL cells and carrier T cells of HTLV-1 simultaneously by allo-HSCT.

Recently, we reported that allo-HSCT would bring about graft-versus-ATLL (GvATLL) effect even without clinically obvious graft-versus-host disease (GVHD).[10] GvATLL could be achieved when a specific immune response targeting HTLV-1 was initiated, such as cytotoxic T cells for tax protein as Harashima

et al.[19] reported. It is also possible that allogeneic immune reaction against recipient cells contributed to GvATLL effect even without HTLV-1-specific immune reactions as seen in transplantations from carrier donors. As most long-term survivors were positive for HTLV-1 provirus and anti-HTLV-1 antibody, our observation suggested that GvATLL had an effect on ATLL cells but not HTLV-1 provirus in most cases. Allogeneic immune reaction without clinically apparent GVHD might be enough to suppress ATLL cells in these situations.

In summary, allo-HSCT for ATLL profoundly reduced provirus load of HTLV-1 in recipients; however, small amounts of HTLV-1-positive cells that remained in long-term survivor were heterogeneous in origin. We also experienced the single case in which HTLV-1 seemed to be eradicated with allo-HSCT. Thus, it was suggested that the way allo-HSCT suppressed and controlled ATLL and HTLV-1 itself was not simple but heterogeneous from case to case. Further analysis is necessary to understand how ATLL is controlled by allo-HSCT through GvATLL effect, and to find how this effect be controlled and enhanced.

Acknowledgements

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Plenary paper

Donor-derived DNA in fingernails among recipients of allogeneic hematopoietic stem-cell transplants

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To examine whether donor-derived cells could exist in nonhematopoietic tissues of recipients after allogeneic hematopoietic stem-cell transplantation, we examined the patterns of the short tandem repeat (STR) of DNA extracted from fingernail clippings of recipients so that the contamination of blood cells was excluded. All 21 patients reached donor-derived hematopoiesis after transplantation and 20 of them were in remission of the primary diseases at the time of sampling. Compared with the STRs of donor cells, among 9 of 21 patients, DNA extracted from fingernail samples showed coexistence of the donor pattern of the STRs, sharing from 8.9% to 72.9% of total STR areas. Time from transplantation to sampling was from 305 to 2399 days among positive cases. These results demonstrate for the first time the existence of stable contribution of donor cells in fingernails among recipients of allogeneic hematopoietic stem cells. (Blood. 2007;110: 2231-2234)

Introduction

Among recipients of allogeneic hematopoietic stem cells (allo-HSCs), donor-derived cells in nonhematopoietic tissues have been observed by several groups.[1-4] For example, in biopsy specimens such as skin, gastrointestinal mucosa, or liver that were taken mostly for the diagnosis of graft-versus-host disease (GVHD), cells carrying the Y chromosome were shown in nonblood cells (eg, epithelium of gastrointestinal tract) of female recipients who received transplants from male donors. These observations and reports of other tissues demonstrated that the cells existed in bone marrow or mobilized peripheral blood that could differentiate into (or contribute to) cells in organs or tissues other than those of hematopoietic lineage (eg, gastrointestinal mucosa, skeletal muscle, buccal mucosa, liver, and skin, etc).[1-7] On the other hand, there were also reports against the contribution, at least major contribution, of donor cells in nonhematopoietic cells in human and animal models.[8-10] One of the reasons for the discrepancy of these reports seemed to originate from methodological limitations.[8,11] For example, in histologic analysis using a fluorescent DNA probe technique such as fluorescence in situ hybridization (FISH), coexistence of blood cells in the tissue samples made it difficult to distinguish true donor-derived cells from recipient cells that 3-dimensionally overlapped the donor-derived blood cells. If samples are taken from a biopsy specimen for the diagnosis of GVHD, they are small in general, which makes it difficult to perform a full analysis of chimerism. To overcome these problems and to examine the existence of donor-derived cells in nonhematopoietic tissue, we investigated the contribution of donor-derived cells in fingernails, the tissue without blood cells, among recipients of allo-HSCs. This is the first report to examine the donor-derived DNA in fingernails.

Patients, materials, and methods

The protocol of this study was approved by the Internal Review Board of Nagasaki University. Twenty-one recipients of allo-HSCs participated in this study and were treated and followed at the Department of Hematology, Nagasaki University Hospital. To examine the existence of donor-derived cells in fingernails, the short tandem repeat (STR) of genomic DNA extracted from fingernail clippers was compared with STRs of donor and recipient cells. After obtaining written informed consent in accordance with the Declaration of Helsinki, fingernail samples of 10 fingers (21 cases) and peripheral blood of participants (9 cases) were collected. A new nail cutter was used for every collection of fingernail samples in each patient to avoid contamination. All pieces of nail from each patient were subjected to extraction of total genomic DNA with proteinase K and phenol/chloroform. From nonseparated total peripheral blood, total genomic DNA was extracted and used to examine the chimerism of hematopoiesis at the time of fingernail sampling in 9 cases. The STR of 10 regions was determined using AmpF*l*STR SGM Plus (Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) was performed using GeneAmp PCR system 9600 (Applied Biosystems), and the difference of STRs was detected using an ABI PRISM 310 Genetic Analyzer and Genotyper software version 2.5 (Applied Biosystems). These procedures followed the instructions of the manufacturer. The contribution of donor-derived cells was described as a percentage of an average of donor peak areas divided by total peak areas of every different STR site (except for sex chromosome). The minimum sensitivity of this method was 5%. The Mann-Whitney *U* test (age and time from HSC transplantation [HSCT] to sampling) and the chi-square test (other factors) were used to test the statistical relationship with the existence of chimerism in nail.

Results and discussion

Table 1 shows the characteristics of donors and recipients, transplantation-related factors, and the percentages of donor-derived DNA in fingernails. All cases demonstrated complete donor chimerism in

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Table 1. Characteristics of hematopoietic stem-cell transplant recipients

Case no.	Diagnosis	Age at HSCT, y	Type of donor	HLA allele compatibility	Conditioning regimen for HSCT	Source of HSCs	aGVHD	cGVHD	Time from HSCT to sampling, d	cGVHD at sampling	Immunosuppressive therapy at sampling	Percentage of donor area
1	ALL	26	Unrelated	Match	CSI+CY+TBI	BM	II	Limited	521	Negative	None	17.2
2	AML	53	Related	Match	BU+CY	BM	None	Limited	2529	Positive	None	0
3	AML	42	Related	Match	BU+CY	BM	II	Limited	569	Negative	None	0
4	AML	23	Unrelated	Match	TBI+CY	BM	None	Limited	1259	Negative	None	39.2
5	AML	45	Related	Match	BU+CY	BM	I	Limited	1657	Negative	None	23.8
6	MDS	18	Related	Match	BU+CY	PB+BM	None	Extensive	1811	Positive	CsA	0
7	SAA	23	Related	Match	BU+CY	BM	I	Extensive	2287	Positive	FK	15.1
8	CML	25	Related	Match	BU+CY	PB	I	Extensive	2252	Positive	PSL	0
9	ALL	55	Unrelated	2 locus mismatch	TBI+Flu+L-PAM	CB	I	Limited	467	Negative	None	0
10	ALL	17	Related	Match	TBI+CY	BM	I	Extensive	999	Positive	CsA+PSL	72.9
11	AML	45	Unrelated	1 locus mismatch	TBI+CY	BM	None	Limited	550	Positive	None	0
12	AML	33	Unrelated	Match	CSI+CY+TBI	BM	II	Extensive	1711	Negative	None	0
13	AML	26	Related	Match	BU+CY	BM	None	Extensive	305	Positive	CsA+PSL+MMF	8.9
14	AML	30	Related	Match	BU+CY	BM	II	Extensive	1580	Positive	FK+PSL+MMF	0
15	ALL	20	Related	Match	BU+CY	BM	I	Limited	1111	Negative	None	69.1
16	ATL	40	Related	Match	TBI+CY	PB	None	Extensive	1518	Positive	CsA	31.2
17	AML	49	Unrelated	Match	TBI+CY	BM	None	Extensive	426	Positive	FK+PSL	0
18	ALL	28	Unrelated	4 locus mismatch	CY+Flu+BU	CB	II	Extensive	851	Positive	None	0
19	AML	17	Related	Match	TBI+CY	PB	II	Extensive	2107	Positive	FK	0
20	AML	42	Related	Match	BU+CY	PB	I	Extensive	1209	Positive	FK+PSL	0
21	CML	46	Related	Match	BU+CY	BM	None	Limited	2399	Negative	None	56.5

ALL indicates acute lymphoblastic leukemia; CSI, craniospinal irradiation; CY, cyclophosphamide; TBI, total body irradiation; BM, bone marrow; AML, acute myelogenous leukemia; BU, busulfan; MDS, myelodysplastic syndrome; PB, peripheral blood; CsA, cyclosporine A; SAA, severe aplastic anemia; FK, tacrolimus; CML, chronic myelogenous leukemia; PSL, prednisolone; Flu, fludarabine; L-PAM, melphalan; CB, cord blood; MMF, mycophenolate mofetil; and ATL, adult T-cell leukemia.

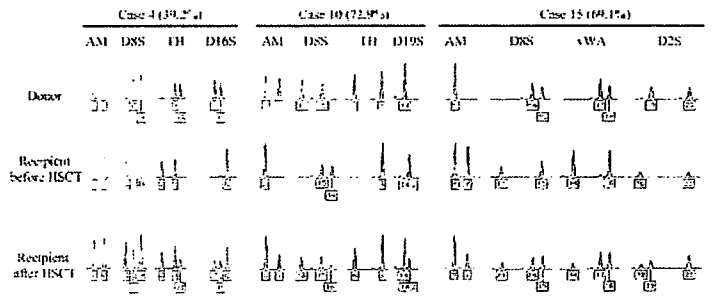

Figure 1. Representative STR patterns of donor blood cells (top), those of recipients before HSCT (middle), and those of nails in recipients after HSCT (bottom) are shown (cases 4, 10, and 15). The percentages in parenthesis were calculated by dividing the donor-derived short tandem repeat (STR) areas by the total STR peak areas in nails of recipients. The numbers of the fragment repeats are indicated below each STR. AM indicates amelogenin gene; D8S, D8S1179; TH, TH01; D16S, D16S539; D19S, D19S433; vWA, von Willebrand factor intron A; and D2S, D2S1338.

bone marrow cells within 100 days from transplantation, and there was no unexplained cytopenia in 17 cases. Among 4 cases (cases 2, 3, 11, and 21) that experienced relapse of primary diseases, 3 of them were in complete donor-derived hematopoiesis at the time of sampling. No case showed abnormal appearance of fingernails, including bleeding, when collected. In case 2, which later died of primary disease, fingernails were collected during partial remission. The other 20 patients were alive and in complete remission when this manuscript was written. In 9 of 21 cases, donor-derived STR peaks were detected in DNA samples of fingernails that shared from 8.9% to 72.9% of total peak areas. Representative STR peaks are shown in Figure 1. There was no statistically significant relationship between the presence of donor-derived STR peaks with mismatch of sex, disparity of blood type, or all factors listed in Table 1. However, because of the small number of cases in this study, we could not deny any relationship among the factors above. The only case that underwent transplantation after reduced-intensity conditioning showed no donor-derived STR in fingernails. So far there is 1 report that used PCR-based genotyping to show the positive contribution of donor-derived cells in nonhematopoietic tissue, buccal epithelial cells.[4] However, the detection and the comparison of STRs of blood or bone marrow samples are established and widely accepted methods for the analysis of chimerism after allo-HSCT, and fingernails have also been used to extract DNA for this type of analysis.[12,13] We did not observe any other peaks than those of recipients or donors in any STR test, demonstrating no contamination of other DNA samples. Taking advantage of the integration of HTLV-1 in T cells among adult T-cell leukemia/lymphoma (ATLL) patients, the existence of HTLV-1 in DNA of fingernails was examined by the genomic PCR of the pX region among 9 ATLL patients, resulting in no amplification in all cases (Figures S1, S2, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). It suggested that no white blood cell (WBC)–derived DNA existed in DNA extracted from fingernails (data not shown). We believe there was little chance of false-positive results in our analysis.

The percentages of donor-derived STR areas were correlated to other reports,[1-4] even a long time after transplantation, demonstrating the stable contribution of donor-derived cells in fingernails. Since nail is a tissue that regenerates continuously throughout life maintained by the stem cells of the nail matrix, the stable contribution of donor-derived DNA in nails suggested the existence of donor-derived cells in the stem-cell system of nails. Based on the clinical observation, when patients are treated with myeloablative conditioning regimen for transplantation, nail stem cells would be damaged to a certain extent so that transient growth retardation or arrest occurs. This harmful condition might facilitate the high contribution of donor-derived cells to nails.[14] In our approach, there is no data available regarding how donor-derived cells resided in these patients: donor-derived nail stem cells existed or the fusion of certain donor cells occurred to nail stem cells of the recipients. We also have no data on the difference of the contribution of donor cells in each finger.

Recently, the absence of donor-derived cells was reported in the hair bulb of allo-HSC recipients (115 cases) where there was no contamination of blood cells either.[9] In spite of the similar biologic features of hair and nail such as continuous regeneration or the same origin from the ectoderm, the contribution of donor-derived cells was clearly different between these 2 tissues after allo-HSCT. There is no clear explanation for the difference; however, biologic characteristics of stem cells and niche circumstances might contribute to these results. This report for the first time demonstrated the long-term, stable contribution of donor-derived cells in nails among recipients of allo-HSCs.

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Authorship

Contribution: D.I., H.S., and R.Y. performed experiments with H.T., and Y.S. and J.T. collected samples. T.F., S.Y., and T.H. collected clinical data. Data were analyzed by D.I. and Y.M. Y.M. organized this study and wrote the manuscript with M.T.

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