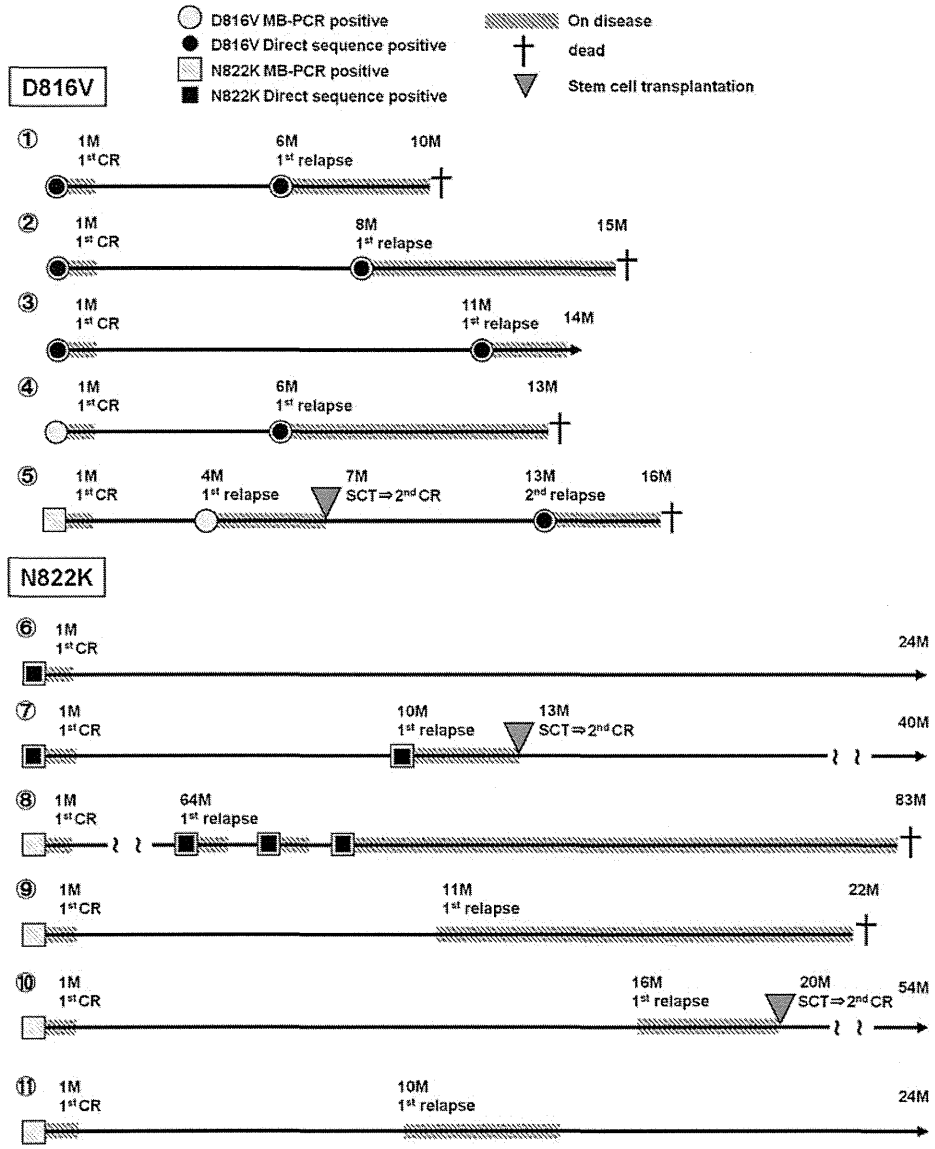

Figure 3 Clinical courses of patients with *c-kit* mutations D816V and N822K. The top panel shows five cases, in which the D816V mutation was observed; the bottom panel shows six cases, in which the N822K mutation was observed. For cases 4 and 5 of D816V and case 8 of N822K, mutations were detected with MB-PCR at an earlier time point than with the DS method. All five cases with detected D816V relapsed, at which time the mutation could be detected even with the DS method. In contrast, among the six cases for which N822K was detected, long-term remission was obtained in one case with chemotherapy alone (number 6). For three cases (number 9, 10 and 11), the N822K mutation was detected at initial presentation but not at relapse. CR, complete remission; M, month; SCT, stem cell transplantation.

Multivariate analyses

We carried out multivariate analyses with the backward stepwise model selection procedure on age (≧60 or <60 years old), gender (male or female), additional chromosomal aberrations (yes or no), WBC count at initial examination (≧20 000/μl or <20 000/μl), hemoglobin (≧10.0g/dl or <10.0g/dl), and post-remission therapy (BHAC-based or HDAC-based) in order to establish the significance of *c-kit* mutations as prognostic factors for relapse of t(8;21)AML.

As a result, *c-kit* mutations and hemoglobin level at initial presentation were extracted as independent events by the backward stepwise model selection. Multivariate analyses with Cox proportional hazards revealed that MB-PCR detection of *c-kit* mutations (hazard ratio 5.074, $P=0.006$, 95%CI: 1.609–16.003) and *c-kit* mutation and/or *Flt3 ITD* (hazard ratio 6.650, $P=0.003$, 95%CI: 1.948–22.695) were independent adverse prognostic factors. On the other hand, multivariate analyses revealed that hemoglobin level at initial presentation were not independent adverse prognostic factors ($P=0.11$).

Discussion

We present MB-PCR as a highly sensitive method for detecting gene mutations and showed that cases for which *c-kit* mutations were observed only at relapse using DS actually already harbored those mutations at initial presentation at a low level undetectable by DS. In this investigation, although we did not

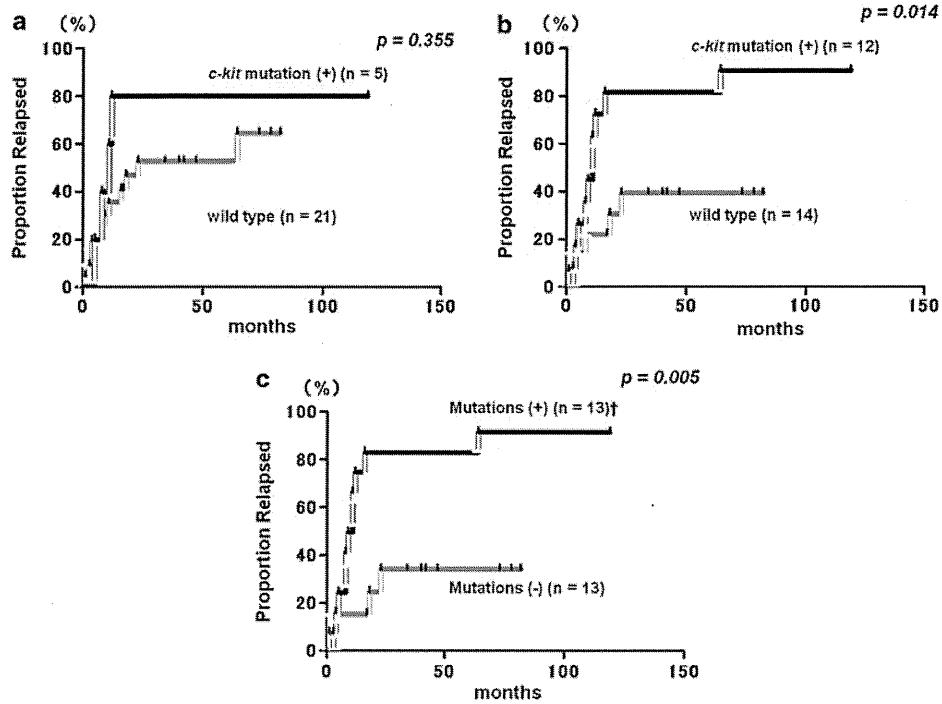

Figure 4 Relationship between presence of *c-kit* mutations and relapse. (**a**) Relationship between the presence of *c-kit* mutations and relapse with the DS method; (**b**) Relationship between the presence of *c-kit* mutations and relapse with the MB-PCR method; (**c**) Relationship between the presence of *c-kit* mutations with the MB-PCR method and *Flt3 ITD*, and relapse. †; *c-kit* mutation ($n=11$), *Flt3 ITD* ($n=1$), double positive ($n=1$).

observe significant differences in relapse rates between cases with and without *c-kit* mutations using the DS method, we did observe a significant difference between cases with and without *c-kit* mutations using MB-PCR (Figures 4a and b). From the above, we conclude that high sensitive method of detecting *c-kit* mutation, such as MB-PCR method, is important to predict prognosis for t(8;21) AML.

These results suggest that a minor leukemia clone with *c-kit* mutation at initial presentation has resistance to treatment and becomes involved in relapse. There have been several recent reports of the role of leukemic stem cells in relapse of leukemia.[39–44] The rare *c-kit* mutation-harboring leukemic cells at initial presentation thought to be involved in relapse may represent leukemic stem cells with *c-kit* mutations. In the future, we plan to analyze *c-kit* mutations in leukemic stem cell fractions using MB-PCR.

Aberrant *c-kit* in t(8;21) AML has been reported in the extracellular domain of exon 8, the juxtamembrane domain of exons 10 and 11, and the A-loop domain with tyrosine kinase activity of exon 17. Some previous *in vitro* studies report that the D816V mutation confers higher tumor growth and antiapoptotic potential compared with mutations in the extracellular domain of exon 8 or in the juxtamembrane domains of exons 10 and 11.[45,46] Similar observations have been reported for *Flt3* mutations, which are class I mutations as found in *c-kit*. Specifically, mutations in the *Flt3* tyrosine kinase domain (*TKD*) confer lower tumor growth and antiapoptotic potential compared with *Flt3 ITD*.[47,48] These findings suggest that biological functions of *c-kit* mutations differ depending on the mutation site, which may affect responsiveness to treatments. In our study, we did not observe any mutations apart from D816V and N822K in the exon 17 A-loop. However, all cases with D816V at initial presentation eventually relapsed while preserving the D816V mutation. In contrast, only one of three cases with N822K at initial presentation were detected N822K at relapse. This result suggests that D816V and N822K may differ functionally, even if this mutation is also in the same A-loop. Thus, we believe that functional analysis of both mutations will be necessary.

Although rare, mutations other than D816V, N822K, and the small T417-D419 deletion have been reported for t(8;21) AML. However, we detected no mutations other than D816V and N822K by screening exons 8–11, 17 and 18 using the both MB-PCR and DS method in relapse samples. On the basis of this, we conclude that at least for the cases examined in our study, *c-kit* mutations other than D816V and N822K were not major for relapse. In the future, we hope to investigate more cases and determine the clinical significance of rare *c-kit* mutations.[20,27,49]

This investigation showed that aberrant *c-kit* at initial presentation in t(8;21)AML is associated with a significantly higher rate of relapse and shorter period of first remission. However, aberrations of unknown gene are involved in t(8;21) AML based on the following observations: a class I aberration must additionally occur in chimeric protein AML1-ETO for t(8;21)AML mouse model onset,[50,51] *c-kit* mutations and *Flt3 ITD* are not observed in about 50% of t(8;21) AML, and there are cases in which the N822K mutation disappeared at relapse. In addition, given report of positive responsiveness to high-dose Ara-C therapy and favorable prognosis of t(8;21) AML with *N-Ras* mutations, which is also a class I mutation,[52] other class I mutations should also be examined.

Our results do not directly suggest that allogeneic SCT is indicated during the initial remission period for t(8;21) AML with *c-kit* mutations, as is the case with AML groups with poor prognosis. Recent reports have been inconclusive as to whether prognosis of t(8;21) AML after the first relapse is poor, like other relapsed AMLs,[15] or still favorable.[53] However, as these results

were not stratified according to *c-kit* mutations, it remains unclear whether allogeneic SCT is indicated during the first remission period for t(8;21) AML with *c-kit* mutations. In addition, we have previously shown the utility of SCT using autologous disease-free peripheral blood stem cells in the first remission period of t(8;21) AML, including cases of *c-kit* mutations at initial presentation.[31] This result is intriguing because it suggests that autologous peripheral blood stem cells without residual disease are useful against t(8;21) AML with *c-kit* mutations. Taking the above into consideration, future studies should investigate stratification of SCT indications during the first remission period according to genetic mutations such as *c-kit* mutations and *Flt3 ITD* for t(8;21) AML, similar to current investigations for normal karyotype AML.

Finally, this is a retrospective study, and the number of patients examined was insufficient to carry out prognostic analysis. A large-scale prospective study is needed to clarify the utility of high-sensitivity analysis of *c-kit* mutations for t(8;21) AML in the clinical setting.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

A single nucleotide polymorphism of IL-17 gene in the recipient is associated with acute GVHD after HLA-matched unrelated BMT

JL Espinoza[1], A Takami[1], M Onizuka[2], T Kawase[3], H Sao[4], H Akiyama[5], K Miyamura[6], S Okamoto[7], M Inoue[8], S Ohtake[1], T Fukuda[9], Y Morishima[10], Y Kodera[11] and S Nakao[1], for the Japan Marrow Donor Program

[1]*Department of Hematology and Oncology, Kanazawa University Hospital, Kanazawa, Japan;* [2]*Department of Hematology and Oncology, Tokai University School of Medicine, Isehara, Japan;* [3]*Division of Epidemiology, Aichi Cancer Center Hospital, Nagoya, Japan;* [4]*Department of Hematology, Meitetsu Hospital, Nagoya, Japan;* [5]*Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan;* [6]*Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan;* [7]*Division of Hematology, Department of Medicine, Keio University School of Medicine, Tokyo, Japan;* [8]*Department of Hematology and Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan;* [9]*Hematopoietic Stem Cell Transplantation Unit, National Cancer Center Hospital, Tokyo, Japan;* [10]*Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan and* [11]*Department of Promotion for Blood and Marrow Transplantation, Aichi Medical University, Nagoya, Japan*

IL-17 has an important role in the host defense against extracellular pathogens and the pathophysiology of autoimmune diseases. This study retrospectively examined the impact of a single-nucleotide polymorphism (rs2275913, G197A) in the IL-17 gene of a total 510 recipients with hematologic malignancies and their unrelated donors on the clinical outcomes in HLA-matched myeloablative (discovery study) and nonmyeloablative (validation study) BMT through the Japan Marrow Donor Program (JMDP). In the discovery study, the presence of a 197A genotype in the recipient resulted in a higher incidence of grades II–IV acute GVHD (hazard ratio (HR), 1.87; 95% confidence interval (CI), 1.23–2.85; $P=0.004$). The donor IL-17A genotype did not significantly influence the transplant outcomes. The validation study showed a trend toward an association of the recipient 197A genotype with an increased risk of grades III–IV acute GVHD (HR, 5.84; 95% CI, 0.75–45.72; $P=0.09$), as well as a significantly increased risk for chronic GVHD (HR, 3.86; 95% CI, 1.29–11.59; $P=0.02$). These results suggest an association of the 197A genotype in the recipient side with the development of acute GVHD.

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Keywords: IL-17; unrelated donor; single-nucleotide polymorphism

Correspondence: Dr A Takami, Department of Hematology and Oncology, Kanazawa University Hospital, 13-1 Takaramachi, Kanazawa 920-8641, Japan.
E-mail: takami@med3.m.kanazawa-u.ac.jp
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Introduction

Hematopoietic SCT represents a therapeutic approach that can potentially cure many patients with otherwise fatal hematologic malignancies. However, its utility is limited because of transplant-related life-threatening complications including GVHD, infections and disease relapse.[1] Among these, acute GVHD is the main cause of early mortality and morbidity. Although HLA matching represents the major genetic determinant in clinical outcome after allo-SCT, recent evidence suggests that non-HLA immune-associated genes are also implicated.[2] Previous investigations have revealed that several single-nucleotide polymorphisms (SNPs), which impact on individual immune response to infections and inflammatory reactions are associated with SCT outcomes including the risk of acute GVHD.[3–12]

IL-17, also known as IL-17A, is the hallmark cytokine of a new T-helper subset termed Th17.[13–16] γδT cells, macrophages and neutrophils are sources of IL-17 as well.[17,18] IL-17 receptor (IL-17RA), a ubiquitous type-I membrane glycoprotein, is expressed in particularly high levels in hematopoietic tissues.[13,19,20] IL-17 has important roles in bridging innate and adaptive immunity, and is involved in the host defense against extracellular pathogens, the pathophysiology of autoimmune diseases, and allograft rejection of solid organs.[21–29] Moreover, several reports have so far shown that Th17 cells and IL-17 has a significant impact on the development of acute GVHD in mouse models.[30–35]

Recent reports have shown association of SNPs in the IL-17 gene with autoimmune diseases such as rheumatoid arthritis and ulcerative colitis.[36–39] The promoter SNP of the IL-17 gene, rs2275913 (G197A), was found to be associated with the susceptibility of rheumatoid arthritis in the Norwegian population[38] as well as that of ulcerative colitis in the Japanese population.[36] The finding that GVHD mimics some aspects of autoimmune diseases prompted us to investigate the impact of donor and recipient SNPs in the IL-17 gene (rs2275913,

G197A) on the clinical outcomes in patients following allogeneic myeloablative BMT using an HLA allele-matched unrelated donor. The data herein show that the presence of the 197A allele in the recipient is associated with a significantly higher incidence of acute GVHD.

Design and methods

Patients

In a total 510 recipients with hematologic malignancies and their unrelated donors on whom IL-17 genotyping was performed, 360 recipients in the discovery study cohort received myeloablative transplantation between January 1993 and July 2002, and 150 recipients in the validation study cohort received nonmyeloablative transplantation between January 1996 and December 2007. Transplantation was undertaken through the Japan Marrow Donor Program (JMDP) with T-cell-replete marrow from an HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 allele-matched donor. HLA genotypes of patient and donor were determined by the Luminex microbead method described previously (Luminex 100 System; Luminex, Austin, TX, USA).[40,41] Although the Luminex microbead method does not provide unambiguous HLA four-digit typing for all genotypes, JMDP has confirmed that this method can identify all HLA alleles with >0.1% frequency among the Japanese population.[42] No patients had a history of any previous transplantation. The final clinical survey of these patients was completed by November 1, 2008. Diagnoses were acute myeloid leukemia in 156 (31%), acute lymphoblastic leukemia in 100 (20%), chronic myeloid leukemia in 94 (18%), myelodysplastic syndrome in 79 (15%), malignant lymphoma in 71 (14%), and multiple myeloma in 10 (2%; Table 1). The recipients were defined as having standard risk disease if acute myeloid leukemia and acute lymphoblastic leukemia were in first CR, malignant lymphoma was in any CR and chronic myeloid leukemia was in any chronic phase and myelodysplastic syndrome. All others were designated as high-risk disease. The myeloid malignancies include acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndrome, and the lymphoid malignancies included acute lymphoblastic leukemia and malignant lymphoma. CYA- or tacrolimus-based regimens were used in all patients for GVHD prophylaxis and anti-T-cell therapy such as anti-thymocyte globulin and *ex vivo* T-cell depletion was not. All patients and donors gave their written informed consent to participate in molecular studies of this nature according to the declaration of Helsinki at the time of transplantation. The project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and JMDP.

IL-17 G197A genotyping

Genotyping of IL-17 was performed using the TaqMan-Allelic discrimination method[43] with a 7900-HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and results were analyzed using the Allelic Discrimination software program (Applied Biosystems). The genotyping assay was conducted in 96-well PCR plates. The amplification reaction contained template DNA, TaqMan universal master mix and the specific probe rs2275913 designed for SNP of IL-17 G197A (product No. C_15879983_10; Applied Biosystems).

Data management and statistic analysis

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after transplantation. Pretransplant CMV serostatus was routinely tested for only patients but not for their donors. Engraftment was confirmed by an ANC of more than 0.5×10^9/L for at least 3 consecutive days. Acute- and chronic GVHD were diagnosed and graded using established criteria.[44,45] The OS was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Transplant-related mortality was defined as death without relapse. Any patients who were alive at the last-follow-up date were censored. The data on causative microbes of infections and postmortem changes in cause of death, as well as the data on supportive care including infections prophylaxis and therapy of GVHD, which were given on institution basis, were not available in this cohort. The analysis was performed using the Excel 2007 (Microsoft Corp, Redmond, WA, USA), OriginPro version 8.0J (Lightstone Inc., Tokyo, Japan) and R (The R Foundation for Statistical Computing, Perugia, Italy) software programs.[46] The probability of OS was calculated using the Kaplan–Meier method and compared using the log-rank test. The probabilities of transplant-related mortality, disease relapse, acute GVHD, chronic GVHD and each cause of death were compared using the Grey test[47] and analyzed using the cumulative incidence analysis,[46] considering relapse, death without disease relapse, death without acute GVHD, death without chronic GVHD and death without each cause as respective competing risks. The variables were recipient age at time of transplantation, sex, CMV serostatus before transplantation, disease characteristic (disease type, disease lineage and disease risk at transplantation), donor characteristics (age, sex, sex compatibility and ABO compatibility), transplant characteristics (TBI-containing regimen, tacrolimus vs CYA and total nucleated cell count harvested per recipient weight) and the year of transplant. The median was used as the cutoff point for continuous variables. The χ^2-test and Mann–Whitney test were used to compare two groups. The Hardy–Weinberg equilibrium for the IL-17 gene polymorphism was tested using the Haploview program.[5] Multivariate Cox models were used to evaluate the hazard ratio (HR) associated with the IL-17 polymorphism. Covariates found to be significant in univariate analyses ($P \leqslant 0.20$) were included in the models. For both the univariate and multivariate analyses, *P*-values were two-sided and outcomes were considered to be significant with $P \leqslant 0.05$.

Results

Discovery study

Frequencies of the IL-17 genotyping. The IL-17 gene polymorphism was analyzed in 360 unrelated BM donor-myeloablative transplant recipient pairs (Table 1). The genotype frequencies of 197A/A, 197A/G and 197G/G were 16, 46 and 38% in recipients and 14, 51 and 36% in

Table 1 Donor and recipient characteristics

	Discovery study (myeloablative transplantation)				P	*Validation study (nonmyeloablative transplantation)*				P
	Recipient IL-17 genotype					*Recipient IL-17 genotype*				
	197A positive n = 223, 62%		*197A negative* n = 137, 38%			*197A positive* n = *87, 58%*		*197A negative* n = *63, 42%*		
	No.	*Ratio (%)*	*No.*	*Ratio (%)*		*No.*	*Ratio (%)*	*No.*	*Ratio (%)*	
Age, years										
Recipient										
Median	33		29		0.12	53		51		0.99
Range	2–65		1–65			1–70		3–68		
Donor										
Median	34		33		0.11	35		33		0.47
Range	20–51		22–51			21–50		20–51		
Year of transplant										
Median	1998		1998		0.65	2004		2004		0.22
Range	1993–2002		1993–2002			1996–2007		1996–2007		
Donor IL-17 genotype										
197A positive	145	65	87	64	0.77	53	61	40	63	0.75
197A negative	78	35	50	36		34	39	23	37	
Sex, male										
Recipient	136	61	74	54	0.81	61	70	39	62	
Donor	141	63	77	56	0.19	26	30	24	38	
Recipient/donor sex										
Sex matched	138	62	86	63	0.99	62	71	43	68	0.20
Male/female	45	20	27	20		14	16	6	10	
Female/male	40	18	24	18		11	13	14	22	
Disease										
Acute myeloid leukemia	73	33	37	27	0.25	23	26	23	37	0.19
Acute lymphoblastic leukemia	48	22	38	28	0.18	9	10	5	8	0.62
Chronic myeloid leukemia	53	24	31	23	0.80	4	5	6	10	0.23
Myelodysplastic syndrome	25	11	16	12	0.89	26	30	12	19	0.13
Malignant lymphoma	23	10	14	10	0.98	19	22	15	24	0.78
Multiple myeloma	1	0	1	1	0.73	6	7	2	3	0.32
ABO matching										
Match	148	66	88	64	0.35	52	60	40	63	0.65
Major mismatch	38	17	17	12		18	21	16	25	
Minor mismatch	32	14	28	20		21	24	10	16	
Bidirectional	5	2	4	3		4	5	3	5	
Conditioning regimen										
With total body irradiation	177	79	115	84	0.28	53	61	39	62	0.90
Without total body irradiation	46	21	22	16		34	39	24	38	
Pretransplant CMV serostatus										
CMV positive recipient	149	67	98	72	0.35	68	78	53	84	0.36
Missing	26	12	18	13	0.68	8	9	10	16	0.21
GVHD prophylaxis										
With cyclosporine	145	65	91	66	0.71	39	45	26	41	0.66
With tacrolimus	78	35	46	34		48	55	37	59	
TNC $\times 10^8$ *per kg*										
Median	5.7		5.7		0.89	4.2		4.5		0.13
Range	0.1–87.0		0.6–87.0			0.8–74.2		1.3–33		
Engraftment	220	99	136	99	0.59	81	93	59	94	0.89

Abbreviation: TNC = total nucleated cell count harvested.

donors. These were similar to previous reports[38,48] in Japanese populations (15, 52 and 33%, respectively) and Caucasian populations (13, 48 and 39%, respectively), and were in accord with the Hardy–Weinberg equilibrium ($P = 0.91$).

Transplant outcome according to the IL-17 genotype. The median follow-up duration in the cohort was 90 months among the survivors (range 4–171 months), 102 recipients (28%) had relapsed or progressed and 187 (52%) had died. Three patients (1%) died before engraftment.

The transplant outcomes according to the IL-17 genotype are summarized in Table 2. The presence of the 197A genotype in the recipient was associated with a significantly higher incidence of grades II–IV acute GVHD (37 vs 23%, $P=0.004$; Figure 1a) as well as a trend toward a higher incidence of grades III–IV acute GVHD (16 vs 10%, $P=0.08$; Figure 1b), whereas no significant differences between the 197A/A and the 197A/G genotype in the recipient were seen in incidences of grades II–IV (38 vs 34%, $P=0.69$) and grades III–IV (17 vs 16%, $P=0.96$) acute GVHD. The 197A genotype on the recipient side showed a tendency to increase a risk of mortality of acute GVHD as a primary cause of death (6 vs 2%, $P=0.095$). There were no significant differences in the impact of a 197A in the recipient genotype on OS, transplant-related mortality, relapse, chronic GVHD or extensive chronic GVHD (data not shown). The donor genotype showed no significant effects on either of these variables in addition to acute GVHD (Table 2).

Multivariate analysis. All of the factors found to be significant in univariate analyses were included in the model. The 197A genotype in recipients remained statistically significant in the multivariate analyses for the development of grades II–IV acute GVHD (Table 3). The presence of a 197A genotype in the recipient side resulted in a higher incidence of grades II–IV acute GVHD (HR, 1.87; 95% confidence interval (CI), 1.23 to 2.85; $P=0.004$) when adjusted for the other factors in the models. In the combined patient group of acute lymphoblastic leukemia and acute myeloid leukemia, this effect was also positive and was close to statistical significance (HR, 1.84; 95% CI, 0.98–3.43; $P=0.056$).

Validation study

The characteristics of the patients in the validation study were similar to those of the patients in the discovery study except for conditioning regimen and recipient age (Table 1). The univariate analysis showed a significant association between the recipient 197A genotype and a higher incidence of grades III–IV acute GVHD (15 vs 4%, $P=0.04$; Figure 1d), whereas no significant difference in the incidence of grades II–IV acute GVHD (33 vs 26%, $P=0.37$; Figure 1c). In the multivariate analysis, the validation study performed on nonmyeloablative SCT did not confirm the association of recipient 197A with grades II–IV acute GVHD found in the discovery study, although there was a trend toward an association with grades II–IV acute GVHD (HR, 5.84; 95% CI, 0.75–45.72; $P=0.09$; Table 4). The recipient 197A genotype was associated with a significantly increased risk for chronic GVHD (HR, 3.86; 95% CI, 1.29–11.59; $P=0.02$), although this association was not found in the discovery study.

Discussion

The discovery study on the basis of myeloablative transplantation showed that the IL-17 197A genotype on the recipient side was associated with a higher risk of grades

Table 2 Univariate analysis of the association of IL-17 genotype with clinical outcomes after transplantation in the discovery study

	No.	*5-year OS (%)*	P	*5-year TRM (%)*	P	*5-year relapse (%)*	P	*II–IV acute GVHD (%)*	P	*III–IV acute GVHD (%)*	P	*Chronic GVHD (%)*	P
Recipient IL-17A genotype													
197A positive	223	53	0.89	27	0.20	24	0.21	37	0.004	16	0.08	48	0.94
197A negative	137	53		21		31		23		10		48	
Donor IL-17A genotype													
197A positive	232	50	0.13	27	0.09	27	0.93	31	0.71	14	0.70	49	0.66
197A negative	128	56		21		27		34		13		47	

Bold values have statistical significance.

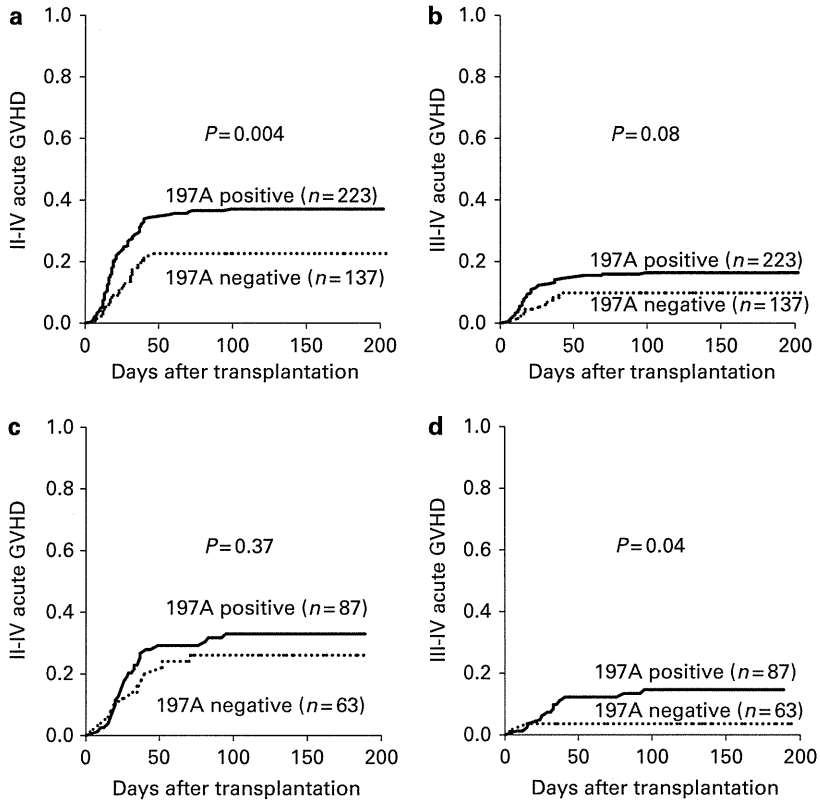

Figure 1 Estimated cumulative incidence curves of grades II–IV (**a**, **c**) and grades III–IV (**b**, **d**) acute GVHD according to the recipient IL-17 genotype in the discovery study (**a**, **b**) and the validation study (**c**, **d**).

Table 3 A multivariate analysis of the association of IL-17 genotype with the clinical outcomes after transplantation in the discovery study

	OS			*TRM*			*Relapse*		
	HR	*95% CI*	P	*HR*	*95% CI*	P	*HR*	*95%CI*	P
197A-positive recipient	0.99	0.72–1.37	0.97	1.00	0.64–1.56	0.99	0.92	0.61–1.37	0.67
197A-positive donor	1.22	0.88–1.71	0.24	1.26	0.79–2.00	0.33	1.04	0.69–1.58	0.85
Recipient age, >30 years	**1.63**	**1.17–2.28**	**0.004**	**2.02**	**1.25–3.28**	**0.004**	—	—	—
Donor age, >32 years	—	—	—	1.29	0.81–2.08	0.29	—	—	—
Female-to-male transplant	—	—	—	1.37	0.82–2.28	0.22	0.76	0.42–1.37	0.36
High-risk disease	**2.02**	**1.47–2.79**	**<0.001**	—	—	—	**2.42**	**1.62–3.61**	**<0.001**
Minor ABO incompatibility	1.19	0.81–1.74	0.38	1.28	0.77–2.15	0.34	—	—	—
CMV-positive recipient	**1.84**	**1.18–3.67**	**0.01**	1.35	0.74–2.48	0.33	—	—	—

	II–IV acute GVHD			*III–IV acute GVHD*			*Chronic GVHD*		
197A-positive recipient	**1.87**	**1.23–2.85**	**0.004**	**1.69**	**0.90–3.22**	**0.10**	0.96	0.69–1.35	0.83
197A-positive donor	0.86	0.59–1.27	0.45	1.13	0.60–1.97	0.70	1.10	0.78–1.55	0.59
Recipient age, >30 years	—	—	—	—	—	—	1.38	0.99–1.93	0.06
Donor age, >32 years	1.41	0.94–2.10	0.10	**2.17**	**1.10–4.23**	**0.02**	1.31	0.92–1.86	0.14
Female-to-male transplant	—	—	—	0.63	0.27–1.49	0.29	—	—	—
High-risk disease	1.32	0.91–1.94	0.15	—	—	—	—	—	—
Minor ABO incompatibility	—	—	—	—	—	—	—	—	—
CMV-positive recipient	—	—	—	—	—	—	—	—	—

Abbreviations: CI = confidence intervals; HR = hazard ratio.
Bold values have statistical significance.

II–IV acute GVHD after unrelated HLA-matched myeloablative BMT through JMDP. The validation study for nonmyeloablative transplantation revealed a trend toward the association of the recipient 197A genotype with an increased risk of grades III–IV acute GVHD, although its association on grades II–IV acute GVHD was unclear. Of

Table 4 A multivariate analysis of the association of IL-17 genotype with the clinical outcomes after transplantation in the validation study

	OS		P	*TRM*		P	*Relapse*		P
	HR	*95% CI*		*HR*	*95% CI*		*HR*	*95% CI*	
197A-positive recipient	0.97	0.55–1.69	0.91	0.92	0.45–1.88	0.82	1.09	0.54–2.20	0.81
197A-positive donor	0.99	0.57–1.71	0.98	0.78	0.39–1.55	0.48	1.52	0.73–3.18	0.26
Recipient age, >52 years	1.63	1.17–2.28	0.004	**2.02**	**1.25–3.28**	**0.004**	—	—	—
Donor age, >32 years	—	—	—	—	—	—	—	—	—
Female-to-male transplant	—	—	—	—	—	—	**3.33**	**1.55–7.13**	**0.002**
High-risk disease	1.21	0.70–2.09	0.49	—	—	—	**2.22**	**1.14–4.30**	**0.02**
Major ABO incompatibility	0.60	0.28–1.27	0.18	—	—	—	—	—	—
Minor ABO incompatibility	0.85	0.43–1.67	0.63	—	—	—	—	—	—
CMV-positive recipient	**5.45**	**1.30–22.87**	**0.02**	6.98	0.94 51.93	0.06	—	—	—
TNC, >4.3 × 10⁸ per kg									
GVHD prophylaxis with tacrolimus	—	—	—	—	—	—	2.04	1.00–4.13	0.049

	II–IV acute GVHD			*III–IV acute GVHD*			*Chronic GVHD*		
197A-positive recipient	1.42	0.74–2.71	0.29	**5.84**	**0.75–45.72**	**0.09**	**3.86**	**1.29–11.59**	**0.02**
197A-positive donor	1.03	0.55–1.94	0.93	1.12	0.33–3.83	0.86	**0.27**	**0.10–0.74**	**0.01**
Recipient age, >52 years	—	—	—	—	—	—	**0.20**	**0.08–0.53**	**0.001**
Donor age, >32 years	—	—	—	—	—	—	—	—	—
Female-to-male transplant	**2.49**	**1.23–5.04**	**0.01**	—	—	—	—	—	—
High-risk disease	—	—	—	—	—	—	—	—	—
Major ABO incompatibility	0.40	0.15–1.02	0.06	—	—	—	—	—	-
Minor ABO incompatibility	—	—	—	—	—	—	—	—	—
CMV-positive recipient				—	—	—	**0.20**	**0.07–0.60**	**0.004**
TNC, $>4.3 \times 10^8$ per kg				—	—	—	0.48	0.19–1.20	0.12
GVHD prophylaxis with tacrolimus				0.49	0.14–1.68	0.26	0.57	0.22–1.48	0.25

Abbreviations: CI = confidence intervals; HR = hazard ratio.
Bold values have statistical significance.

note, the validation study has demonstrated the association between the recipient 197A genotype and the increased incidence of chronic GVHD. This might reflect the association between the recipient 197A genotype and the risk of late acute GVHD,[49] considering that late acute GVHD occurs frequently after nonmyeloablative conditioning transplantation[50] and that the manifestation of late acute GVHD is usually indistinguishable from chronic GVHD.[51] In this study, the diagnosis of chronic GVHD was based on historical criteria,[45] and data on chronic GVHD classification according to the new NIH criteria[49] were unavailable, thus suggesting that late-onset, prolonged or delayed acute GVHD could have been diagnosed as chronic GVHD. Taken together, it would appear that the validation cohort data is consistent with the discovery cohort data, although additional validation studies are warranted. This is the first report to demonstrate that IL-17 may be involved in the pathophysiology of acute GVHD in humans.

The role of IL-17 in pathogenesis of acute GVHD remains unclear. Several mouse model experiments have revealed that transfer of IL-17-producing cells induced acute GVHD,[33–35] whereas in contrast there is a report[31] showing that donor IL-17-producing cells ameliorated acute GVHD. Host DCs are critical in the initiation of acute GVHD,[52–54] leading to a hypothesis that IL-17-producing cells could modify the function of host DCs through unknown mechanisms. Direct interaction between IL-17 and host DCs may be supported by the fact that DCs expressed IL-17 receptors.[26] As the IL-17 G197A polymorphism is located in the promoter region of IL-17 gene, it is conceivable that it may exert some roles in the transcriptional regulation of IL-17 secretion. Thus, investigating the influence of the IL-17 G197A polymorphism on the expression of IL-17 may offer useful information on this issue.

The current study did not show an association between the risk of acute GVHD and the IL-17 genotype in the donor side, implying an influence of host IL-17-secreting cells such as Th17 cells might be more important than the influence of donor IL-17-secreting cells on the pathophysiology of acute GVHD. However, it is still unclear how IL-17 secreted from the host IL-17-secreting cells is involved in the development of acute GVHD. Patient serum and lymphocytes may offer useful information on this issue, although these samples were not obtained for our study.

This study showed that the increased risk of acute GVHD associated with the host 197A genotype of IL-17 did not significantly benefit those with transplant-related mortality and OS after BMT. This might result from the low incidence of acute GVHD-related mortality regardless of the host IL-17 genotype in this cohort. Further investigations for patients at higher risk for acute GVHD including PBSC or HLA-mismatched transplant recipients should be warranted to clarify this issue.

The discovery study also indentified higher recipient age, high-risk disease and CMV-positive recipient as significant predictive factors for worse transplant outcomes (Table 3), which is consistent with earlier studies.[55–57] In addition, similar to a previous report,[58] higher donor age was associated with the increased risk of grades III–IV acute GVHD, which might result from the replacement of naive T cells by memory T cells with aging.[59]

This study suggests that genotyping of IL-17 in transplant recipients before transplantation may provide a 197A-positive recipient an opportunity to avoid the risk of acute GVHD by favoring a BM or cord blood, and an HLA-matched graft rather than a PBSC or HLA-mismatched graft. However, single polymorphisms in one cytokine gene are unlikely to determine the majority of acute GVHD. Future development of predictive strategies including multiple sets of genes will be required.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Rapid progression and unusual premortal diagnosis of mucormycosis in patients with hematologic malignancies: analysis of eight patients

Shingo Yano · Jiro Minami · Kaichi Nishiwaki · Takaki Shimada · Nobuaki Dobashi · Yuichi Yahagi · Yutaka Takei · Shinobu Takahara · Yoji Ogasawara · Katsuki Sugiyama · Yuko Yamaguchi · Takeshi Saito · Kinuyo Kasama · Hiroki Yokoyama · Tomohito Machishima · Atsushi Katsube · Noriko Usui · Keisuke Aiba

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Abstract Mucormycosis is a rare but emerging group of life-threatening opportunistic mycoses. We described experience of eight patients who developed mucormycosis. These patients had developed hematologic malignancies, and none achieved complete remission. Six of the eight patients presented with neutropenia, five received corticosteroid, and four had concomitant hyperglycemia. The most frequent physical finding was fever, and five patients complained of facial pain, headache, or chest pain. Four patients presented with concomitant bacterial infection, pulmonary aspergillosis, or intestinal candidiasis. Premortal diagnosis of mucormycosis was made in only one patient. Postmortem biopsy or autopsy was the diagnostic tool for the other patients. Although patients who were treated with amphotericin B survived longer than those treated with micafungin or voriconazole, all patients died due to the progression of mucormycosis. Estimated median survival was 23 days. Premortal diagnosis was rarely achieved as biopsy of infected tissues was the only diagnostic tool, and four patients who revealed dual infection were diagnosed with aspergillosis or bacterial infections. In patients with a high risk of mucormycosis presenting with pain and uncontrollable fever, mucormycosis should be included in the differential diagnosis. High dosages of liposomal amphotericin B should be given and surgical debridement should be performed promptly in cases highly suggestive of mucormycosis.

Keywords Mucormycosis · Hematologic malignancies · Liposomal amphotericin B · Dual infection

S. Yano (✉) · J. Minami · K. Nishiwaki · T. Shimada · N. Dobashi · Y. Yahagi · Y. Takei · S. Takahara · Y. Ogasawara · K. Sugiyama · Y. Yamaguchi · T. Saito · K. Kasama · H. Yokoyama · T. Machishima · A. Katsube · N. Usui · K. Aiba
Division of Clinical Oncology and Hematology, Department of Internal Medicine, Jikei University School of Medicine, 3-25-8 Nishi Shinbashi Minato-ku, Tokyo 105-8461, Japan
e-mail: yano@jikei.ac.jp

1 Introduction

Invasive fungal infections (IFIs) have emerged as important causes of morbidity and mortality in patients with hematologic malignancies, particularly in patients with prolonged neutropenia after chemotherapy. Although the primary causative organisms are candidiasis and aspergillosis, unusual organisms are recovered with increasing frequency. Distinctive members of the order mucormycosis (zygomycosis) are rare but emerging life-threatening opportunistic mycoses. The incidence of mucormycosis is 1.7 cases per million people per year [1]. In patients with a high risk, such as those undergoing allogeneic hematopoietic transplantation, the incidence of mucormycosis has been described as 2–3% [2]. The name zygomycosis is derived from the development of spores of these fungi during sexual reproduction that look like the zygomatic arch. Mucormycosis species include *Rhizopus*, *Rhizomucor*, *Absidia*, and *Cunninghamella*.

Mucormycosis are ubiquitous and saprophytic fungi that can be found in the environment. For example, *Rhizopus* and *Rhizomucor* are commonly found in decaying food, old bread, and soil; other typical sources are plants and construction sites [3–5]. Although normal immunocompetent hosts rarely develop mucormycosis, the disease typically occurs in patients with diabetes mellitus, patients with malignancy, recipients of organ or hematopoietic transplantation, and recipients of deferoxamine [6–8]. Following

inhalation of airborne spores, primary infection develops in the respiratory tract. Since the actions of neutrophils and phagocytes are the major host defense mechanisms against mucormycosis, patients with neutropenia or dysfunction of phagocytes are at high risk of developing the disease [7].

Clinical manifestation of mucormycosis can be divided into five clinical categories: (1) rhinocerebral, (2) pulmonary, (3) cutaneous, (4) gastrointestinal, and (5) disseminated. These forms of mucormycosis tend to occur in patients with specific risk factors. For example, those with diabetic ketoacidosis typically develop the rhinocerebral form [9], whereas pulmonary infection occurs more often in those with malignancy. Neutropenic patients are at high risk of developing the disseminated form of the disease [10], which is characterized by a high rate of mortality.

The diagnosis of mucormycosis is based on histopathological examination of surgical or biopsied specimens. Microbiological culture is usually negative, and there are no reliable serologic or PCR-based tests for diagnosis of mucormycosis. Biopsy may be the only positive diagnostic tool for most patients.

Cases of mucormycosis in Japan have been described sporadically. We report here eight patients with hematologic malignancies who developed mucormycosis and review the recent literature.

2 Patients and methods

The medical records of patients affected by hematologic malignancies with the diagnosis of mucormycosis between 1997 and 2008 were reviewed retrospectively. The diagnosis of mucormycosis was made on the basis of histopathological examination of tissue biopsy or autopsy specimens. Patient characteristics, type of hematologic disease, clinical symptoms and signs of infection, radiological findings, site of infection, results of treatment, and autopsy or biopsy findings were collected for the analysis of mucormycosis.

Statistical analysis was performed with Dr SPSS II software. The probability of overall survival (OS) was calculated according to the Kaplan–Meier method.

3 Results

3.1 Clinical profile

Among 3,082 patients with hematologic malignancies who had been treated with chemotherapy at Jikei University Hospital between March 1997 and December 2008, eight patients suffered from mucormycosis. The background of patients is listed in Table 1. Three patients had myelodysplastic syndrome (MDS), two patients had acute myeloid leukemia (AML), one had acute lymphoblastic leukemia (ALL), one had chronic myeloid leukemia (CML), and one had multiple myeloma (MM). None of them achieved complete remission (CR) at the time of the fungal infection. Two patients were under treatment of induction chemotherapy and six patients received salvage therapy or supportive care. Six of eight patients had a neutrophil count below 500/μl and four patients were treated under laminar air flow environment, five of them received corticosteroid, and four patients had concomitant hyperglycemia (Table 1). None of the patients received the iron chelator deferoxamine.

3.2 Clinical manifestations

Clinical forms of mucormycosis were classified into rhinocerebral (1 patient), pulmonary form (2 patient) or disseminated (5 patients) (Table 1). The most frequent symptom was fever. Five of eight patients complained of facial or oral pain, headache, or chest pain. Serum β-D-glucan was elevated in two patients (Case 2, 7) and serum galactomannan antigen was elevated in one patient (Case 6). In accordance with the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [11], definitive premortal diagnosis of proven mucormycosis was made in only one patient (Case 3) using sinus tissue collected by biopsy. The probable diagnosis of aspergillosis was made in three patients using host factor (neutropenia), clinical criteria (ethmoid infiltration on sinonasal CT in Case 2, or dense, well-circumscribed lesions on lung CT in Case 6 and 7), and mycological evidence (detection of galactomannan antigen or β-D-glucan) (Table 1). The possible diagnosis of aspergillosis was made in one patient (Case 1). The other three patients were clinically diagnosed with pneumonia (Case 4), cellulitis (Case 5), or bacterial sepsis (Case 8). In seven out of eight patients, autopsy or postmortem biopsy was the only positive diagnostic tool.

3.3 Outcome

Five patients who were clinically diagnosed with mucormycosis or aspergillosis were treated with amphotericin B or voriconazole, and the other three patients were treated with micafungin (Table 1). A sinus tissue biopsy enabled correct diagnosis of the disease in Case 3, who was treated with amphotericin B and surgical debridement resulting in a longer survival of 6 months. However, the prognosis of mucormycosis disease was poor in our patients and median survival was 23 days (Fig. 1). Although it seems that patients who were treated with amphotericin B survived longer than those with micafungin or voriconazole

Table 1 Clinical characteristic and outcome

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Age/sex	30/M	81/M	67/M	71/M	66/M	33/M	28/M	59/F
Disease (status)	ALL (nonCR)	MDS (RCMD)	MDS (RCMD)	AML (nonCR)	MDS (RAEB-1)	AML (nonCR)	CML (BC)	MM (nonCR)
Type of treatment	Induction	Supportive care	Supportive care	Supportive care	Induction	Salvage	Salvage	Salvage
Neutropenia (<500/μl)	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Steroid exposure	Yes	Yes	No	No	No	Yes	Yes	Yes
Hyperglycemia	No	No	No	Yes	Yes	Yes	Yes	No
Elevation of B-D glucan or GM	No	Yes	No	No	No	Yes	Yes	No
Patterns of mucormycosis	Pulmonary	Dissemianted	Rhinocerebral	Dissemianted	Dissemianted	Dissemianted	Pulmonary	Dissemianted
Symptom of pain	No	Head	Face	No	Oral	Chest	Chest	No
Diagnosis derived	Postmorten biopsy	Autopsy	Surgery	Autopsy	Autopsy	Autopsy	Postmorten biopsy	Autopsy
Therapy	AMPH	AMPH	AMPH	MCFG	MCFG	VRCZ	AMPH	MCFG
Survival (days)	28	34	193	14	18	8	68	23
Mixed infection		Candida				Aspergillus Bacterial	Aspergillus	Bacterial
Autopsy (site of mucormycosis)	Not done	Brain, cardiac ethmoid sinus lung, kidney	Not done	Brain, cardiac, lung, kidney, liver	Brain, cardiac, lung, kidney, liver, gut, aorta, cellutis	Brain, cardiac, lung, liver, aorta	Not done	Lung, gut

M male, *F* female, *AML* acute myeloid leukemia, *ALL* acute lymphoblastic leukemia, *MDS* myelodysplastic syndrome, *RCMD* refractory cytomenia with multillineage dysplasia, *RAEB* refractory anemia with excess of blasts, *CML* chronic myeloid leukemia, *MM* multiple myeloma, *CR* complete remission, *BC* blastic crisis, *GM* galactomannan, *AMPH* amphotericin B, *MCFG* micafungin, *VRCZ* voriconazole

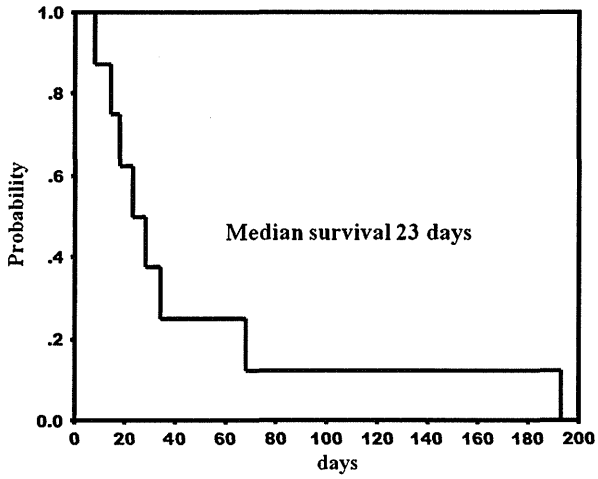

Fig. 1 Probability of survival after episode of mucormycosis. Survival was measured from initiation of uncontrollable fever to death. Estimated median survival was 23 days

(Table 1), all patients eventually died of the progressive disease of mucormycosis.

Radiographic examination demonstrated rapid progression of the disease. One patient (Case 4) had an infiltrate in the right lower lobe although there was no evidence of infiltration 10 days beforehand (Fig. 2a, b). One patient (Case 5) did not have any lesions in the central nervous system (CNS) upon the occurrence of loss of consciousness, but CT scan revealed that marked infiltrate appeared 4 days later (Fig. 2c, d).

Autopsy or postmortem biopsy was carried out on seven patients. The main site of involvement of mucormycosis was lung. Other sites of infection were brain, ethmoid sinus, heart, kidney, liver, and large bowel (Table 1). Although three patients (Case 2, 6, 7) were clinically diagnosed with the probable aspergillosis, autopsy or postmortem biopsy revealed that two patients (Case 6, 7) had both aspergillosis and mucormycosis and one patient (Case 2) had the coexistence of intestinal candidiasis but no evidence of aspergillosis. The infiltrative lesions of lung in Case 6, 7 should be caused by both aspergillosis and mucormycosis, but ethmoid lesion in Case 2 must be caused by mucormycosis. Finally, four patients in the present series revealed the coexistence of other fungus and/or bacterial infection (Table 1). An autopsy specimen obtained from a patient (Case 5) revealed many microscopic hyphae of mucormycosis in an artery (Fig. 3).

4 Discussion

Mucormycosis is a rare but emerging group of life-threatening opportunistic fungal infections. Because the actions of neutrophils and phagocytes are the major host defense mechanisms against mucormycosis, patients with neutropenia or dysfunction of phagocytes are at high risk of developing the disease [7]. Macrophages kill intracellular spores by oxidative mechanisms, whereas neutrophils can damage fungal hyphae by extracellular mechanisms [12]. Although the exact mechanisms involved remain unknown, hyperglycemia and acidosis impair the ability of phagocytes to defend an organism. Corticosteroid exposure also negatively affects the ability of macrophages to prevent germination of the spores in a murine model [13]. As such, the disease typically occurs in patients with hematologic malignancies, recipients of organ or hematopoietic stem cell transplantation, and those with diabetes mellitus. In fact, all our patients had hematologic malignancies, none of them obtained CR, five of them received corticosteroid, and four of them had hyperglycemia. But there must be important mechanisms that initiate the disease, because only a few immunocompromised patients develop mucormycosis. For example, inhaled hyphae may need to undergo some essential process to convert into the infectious spores.

Iron overload should also play a role in the development of mucormycosis [2]. Not only the release of free iron allows rapid fungal growth, but also the iron chelator deferoxamine induces marked increase of mucormycosis [7]. The use of deferoxamine should not be recommended to treat iron overload in patients with a high risk of mucormycosis. On the other hand, a new iron chelator deferasirox did not allow the organism to take up iron and did not support the growth of mucormycosis because it has a higher affinity constant for iron and, as a result, deprives the fungi of iron, inhibiting their growth [14]. In the present cases, none of the patients received deferoxamine. However, it seems that transfusional iron overload might affect the incidence of the disease because patients for whom serum iron was evaluated showed high ferritin levels (median 4,821 ng/ml, range 791–9,349 ng/ml).

Roden et al. [6] reviewed 929 cases of mucormycosis and reported that diabetes type II was the most common underlying disease, followed by no primary underlying disease, and third, hematologic malignancy. The primary site of infection at the time of initial diagnosis varied as a function of the host population. Patients with diabetes mellitus were associated with rhinocerebral mucormycosis, and cutaneous mucormycosis constituted one-half of infections in patients with no underlying disease; patients receiving deferoxamine had a tendency to develop disseminated disease [6, 9]. Pulmonary mucormycosis has been found to occur in patients with hematologic malignancies [6], and pulmonary disease in neutropenic patients has the highest incidence of dissemination [7]. In our patients, affected sites of mucormycosis were rhinocerebral (one patient), pulmonary

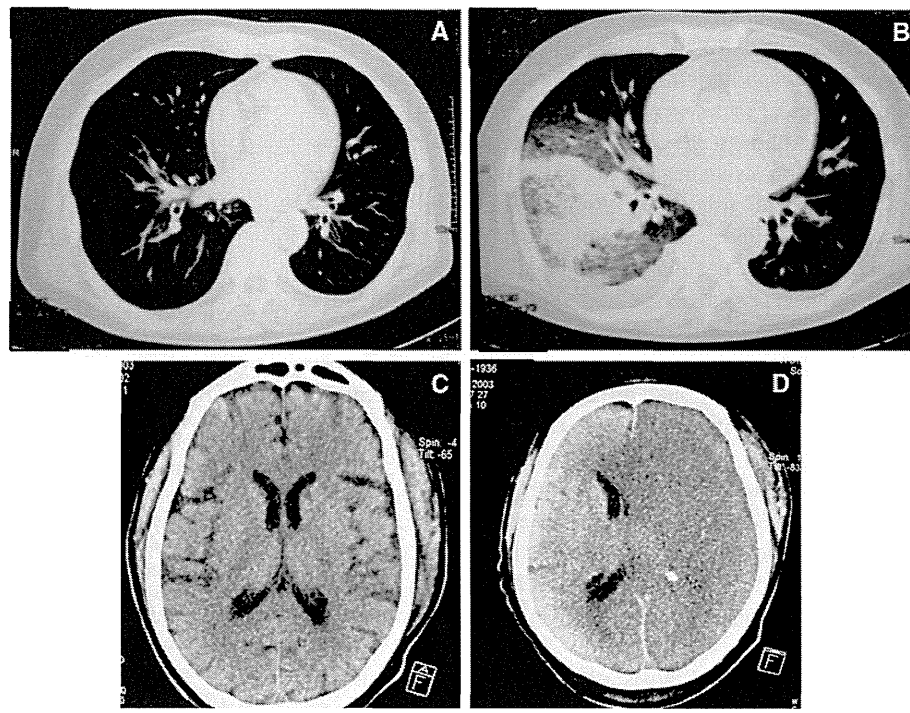

Fig. 2 Computed tomography (*CT*) scan of patient No. 4 shows a cavitated consolidation in the right lung (**b**), although there was no evidence of the infiltration 10 days beforehand (**a**). CT scan of patient No. 5 shows marked infiltrate with edema in the brain (**d**), but the patient did not have any lesions in the central nervous system at an occurrence of loss of consciousness (**c**)

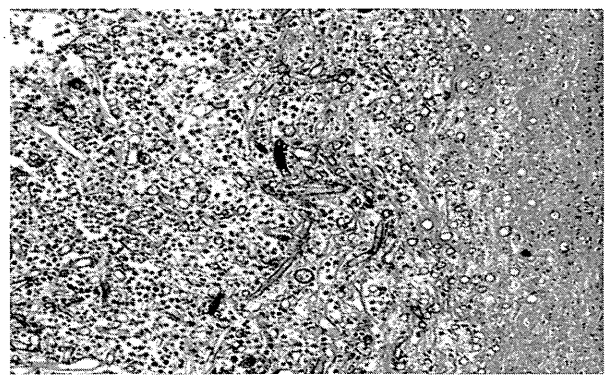

Fig. 3 Hyphae of mucormycosis occupied aortic intima and aortic media on Masson trichrome stain (patient No. 5). Non-septate hyphae of mucormycosis were unbranched

(2 patients), and disseminated (5 patients) (Table 1). Because a postmortem autopsy was not carried out in three patients (Case 1, 3, 7) with rhinocerebral or pulmonary mucormycosis, it is possible that their diseases had already developed into disseminated mucormycosis.

Since there are no specific symptoms and radiographic findings and no reliable serologic or PCR-based tests for mucormycosis, its diagnosis is generally difficult and can only be made pathologically with biopsy specimens of infected tissues. In our experience, the most frequent physical finding was fever and five out of eight patients complained of facial pain, headache, or chest pain; the symptom of pain with uncontrollable fever may be a suggestive but not specific symptom of mucormycosis. We could make correct diagnosis for only one patient (Case 3) using a collected biopsy specimen. The other seven patients were diagnosed with mucormycosis by postmortem needle biopsy or autopsy. Given the difficulty of making premortal diagnosis, there may still be a possibility that the incidence of mucormycosis has been underestimated. Unexpectedly, four patients were found to have dual infection. Findings from our patients suggested that, even when patients are diagnosed with bacterial or fungal infection other than mucormycosis, we should suspect mucormycosis if they do not respond to treatments with antifungal agents and/or antibiotics.

The success of treatment of mucormycosis may require the following four steps: (1) early diagnosis, (2) removal of risk factors such as neutropenia, hyperglycemia, and administration of deferoxamine, (3) surgical debridement, and (4) antifungal therapy [15]. Surgical debridement of pulmonary disease improved survival more than that in patients treated with antifungal therapy alone [16–19]. However, patients with neutropenia usually develop disseminated disease in the early stage and multiple diseases do not allow for surgical resection. For success of surgical treatment, one of the most important issues is early diagnosis because focal lesions can be excised before they progress to disseminate. Therefore, aggressive biopsy should be undertaken.

There have been no prospective randomized trials to define the optimal antifungal therapy for mucormycosis.

However, as amphotericin B remains the only licensed antifungal agent for the treatment of mucormycosis [15], liposomal amphotericin B in the highest tolerable dosage must be one of the most recommended antifungal therapies in Japan. Although the optimal dose of liposomal amphotericin B remains unclear, doses in the range 10–15 mg/kg/day have been used [20]. Another attractive agent for mucormycosis is posaconazole, which is a new broad-spectrum triazole not available in Japan [21]. In neutropenic mice model, posaconazole was statistically less effective than amphotericin B [22]. Therefore, posaconazole has been considered to be a reasonable option for patients with mucormycosis who are refractory to or intolerant of amphotericin B. Itraconazole has limited activity against *Absidia* species, and fluconazole and voriconazole do not have reliable activity against mucormycosis [15, 23–25]. Micafungin and caspofungin, members of the echinocandins, were synergized with amphotericin B lipid complex in treating murine mucormycosis [26]. These agents may have a role as a second agent, especially in combination with the polyene.

In our series, prognosis of mucormycosis disease in patients with hematologic malignancies was poor, and estimated median survival was 23 days. Only one patient who survived longer than 6 months received surgical debridement and administration of amphotericin B at the dosage of 0.5 mg/kg/day. Although patients who were treated with amphotericin B survived longer than those with micafungin or voriconazole, all patients died of the progressive disease. The reason for our poor results was that most patients could not be treated with aggressive surgical debridement, and we should have treated the patients with high dosage of amphotericin B or liposomal amphotericin B.

Mucormycosis is a rare fungal infection, but the mortality rate remains high. Premortal diagnosis was difficult because the biopsy of infected tissues was the only diagnostic tool and some patients revealed dual infection. We conclude that, even when patients are diagnosed with bacterial or fungal infection other than mucormycosis, we should consider the possibility of mucormycosis, in particular if patients have the symptoms of pain with uncontrollable fever. High dosages of liposomal amphotericin B should be given and surgical debridement should be attempted promptly in cases that are highly indicative of mucormycosis.

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