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Importance of *c-kit* mutation detection method sensitivity in prognostic analyses of t(8;21)(q22;q22) acute myeloid leukemia

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Recently, *c-kit* mutations have been reported as a novel adverse prognostic factor of acute myeloid leukemia with t(8;21)(q22;q22) translocation (t(8;21) AML). However, much remains unclear about its clinical significance. In this study, we developed a highly sensitive mutation detection method known as mutation-biased PCR (MB-PCR) and investigated the relationship between *c-kit* mutations and prognosis. When *c-kit* mutations were analyzed for 26 cases of t(8;21) AML using the direct sequence (DS) and MB-PCR, the latter had a much higher detection rate of *c-kit* mutations at initial presentation (DS 5/26(19.2%) vs MB-PCR 12/26(46.2%)). Interestingly for the three cases, in which *c-kit* mutations were observed only at relapse with the DS, *c-kit* mutations were detected at initial presentation using the MB-PCR. This result suggests that a minor leukemia clone with *c-kit* mutations have resistance to treatment and are involved in relapse. In univariate analyses, the presence of a *c-kit* mutation using DS was not an adverse prognostic factor ($P=0.355$), but was a factor when using MB-PCR ($P=0.014$). The presence of *c-kit* mutations with MB-PCR was also an independent adverse prognostic factor by multivariate analyses ($P=0.006$). We conclude that sensitivity of *c-kit* mutation detection method is important to predict prognosis for t(8;21) AML.

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Introduction

Acute myeloid leukemia (AML) has the highest incidence among adult leukemia and includes a variety of subtypes with varying pathology, responsiveness to treatment and prognoses.[1,2] AML with the t(8;21)(q22;q22) translocation (t(8;21) AML), constitutes about 10% of all AMLs and has been considered an AML group with favorable prognosis based on its positive responsiveness to chemotherapy and high rate of complete remission.[3–5] Many studies have reported that when high-dose Ara-C is used as post-remission therapy for t(8;21) AML, prognosis is good with overall disease-free survival rates of 50–70%.[6–11]

However, although t(8;21) AML has good prognosis overall, about 40% of cases relapse, of which half become treatment resistant. In fact, reports suggest that the prognosis of t(8;21) AML following first relapse is just as poor as other relapsed AMLs even when stem cell transplantation (SCT) is performed.[12–15] Stratifying prognoses for t(8;21) AML become critical when determining, for example, SCT indications during the initial remission period. The white blood cell (WBC) count at initial examination,[12] WBC index,[16,17] additional chromosomal aberrations such as del(9)[18] and loss of sex chromosomes and CD56 positivity[19] have been reported as adverse prognostic factors of t(8;21) AML. In recent years, *c-kit* mutations have also been proposed as a novel marker.[20,21]

The *c-kit* gene is located on chromosome 4q11–12 and encodes a 145 kD type III receptor tyrosine kinase. It has five extracellular immunoglobulin-like domains, a juxtamembrane domain and an intracellular kinase domain. *c-kit* mutations have been found in gastrointestinal stromal tumors (70% or more), mastocytosis (90% or more) and germ cell tumors (about 10%).[22,23] In addition, they have been reported in ~12–25% of cases of core-binding factor (CBF)–AMLs such as inv(16)(p13q22) and t(8;21) AML.[24] Normal *c-kit* sends proliferative cues into cells following stimulation by its ligand, stem cell factor and has an important role in early hematopoiesis.[25] On the other hand, mutant *c-kit* send proliferative signals into cells in a ligand-independent manner, and are considered class I aberrations, which have a role in leukemic cell proliferation.[26] In recent years, it has been reported that CBF–AML cases with *c-kit* mutations show a high rate of relapse and poor prognosis.[20,21,27–29] Reflecting these reports, the National Comprehensive Cancer Network's (NCCN) 2009 guidelines considered t(8;21) AML with *c-kit* mutations to be an intermediate prognosis group. However, at present, much remains unclear about the significance of *c-kit* mutations as a prognostic factor for CBF–AML. For example, in inv(16)(p13q22) AML, prognosis of patients with or without mutation in *c-kit* exon 8 is not significantly different.[21] Recently it is also reported that prognosis of pediatric CBF–AML patients with or without *c-kit* mutations is not significantly different.[30]

The sensitivity of the detection method may be one reason why *c-kit* mutations remain an unclear prognostic factor for t(8;21) AML. A large majority of t(8;21) AML cases is classified as M2 by the French–American–British (FAB) Classification. Depending on the case, the proportion of leukemic cells may be less than 50%. In addition, nearly all *c-kit* mutations are heterozygous. It is possible that leukemic cells with *c-kit* mutations are quantitatively small populations at initial presentation and hence, not detectable with the direct sequence (DS) method. If the minor leukemic cells with *c-kit* mutations at initial presentation become treatment resistant and are involved in relapse, then a highly sensitive method to detect *c-kit* mutations is necessary in order to clarify the significance of *c-kit* mutations as a prognostic factor. Therefore, we used a highly sensitive mutation detection method known as mutation-biased

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PCR (MB-PCR) to detect *c-kit* mutations and investigated their significance as a prognostic factor for t(8;21) AML.

Patients and methods

Patient samples

We retrospectively analyzed 26 cases of t(8;21) AML treated at the Nippon Medical School and its affiliated facilities between January 1991 and January 2009. A search for *c-kit* mutations and *Fms-like tyrosine kinase 3 internal tandem duplications* (*Flt3 ITD*) was carried out on 26 initial diagnosis and 15 relapse cases where bone marrow or peripheral blood samples, in which 30% or more of the blast cells were available for use. Informed consents were obtained from the patients for genetic testing. This protocol was approved by our institutional review board.

Mutation analysis for c-kit and Flt3 ITD

Mononuclear cells from bone marrow or peripheral blood were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC, USA). Genomic DNA of mononuclear cells was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). PCR amplification of *c-kit* and *Flt3 ITD* was performed as previously described.[31,32] Mutational analysis of the extracellular domain (exons 8 and 9), transmembrane domain (exon 10), juxtamembrane domain (exon 11) and the second intracellular kinase (TK) 2 domain (exons 17 and 18) of the *c-kit* gene was carried out with PCR followed by direct sequencing. Specific sequences of primers used for PCR and sequencing are available on request. To validate sequencing results, PCR products were inserted into the pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Recombinant plasmids isolated from 8 to 12 white colonies were sequenced.

MB-PCR for c-kit mutations using QProbe

Quenching probe (QProbe) is a fluorescent probe in that a fluorescent substance is bound to cytosine on the terminal portion of the probe, which becomes quenched on hybridization with a complementary strand. With increasing temperature, the duplex unravels at a temperature (Tm) related to the strength of the bond between the QProbe and the complementary chain, at which point the fluorescence intensity recovers. Tm analysis is a technique to determine the degree of complementation between the QProbe and target nucleic acid by measuring the change in fluorescence intensity with increasing temperature.[33,34] Here, we detected *c-kit* mutations using the QProbe method and a prototype of i-densy, a fully automated single nucleotide polymorphism genotyping systems.[34] Amplification was performed with MB-PCR for high-sensitivity detection of mutations using probes that perfectly matched the mutated form. In MB-PCR, the 3′ terminal region of the primer is established as the mutation site, and allele-specific primers for wild-type and mutant primers are used for amplification. By adjusting the length of the primer, we devised a means by which the mutant form could be amplified more than the wild-type form. Furthermore, given the correlation between the proportion of the mutant form and the value of the fluorescence intensity detected by QProbe, it is possible to semiquantify the amount of mutations.

For sensitivity analysis, normal cells and clinical samples with heterozygous mutations were mixed and detection sensitivity was assessed. The ratio of each mutation was controlled by adjusting the ratio of blast cells from the clinical samples. Detection sensitivity was also assessed using a mixture of DNA from a normal subject and *c-kit* mutations containing fragment generated by PCR and TA cloning from clinical samples.

Statistical analysis

Patient characteristics were compared using Chi-square, Fisher and *t* tests. In addition, we analyzed the cumulative incidence of relapse (CIR) using the Kaplan–Meier log-rank test. Prognostic factors for t(8;21) AML were analyzed with multivariate analyses using Cox proportional hazards. In order to extract independent events, those events with a *P*-value of 0.20 or less were analyzed using the backward stepwise model selection procedure. Statistical analyses were carried out using SPSS software (version 12.1.4; SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of patients with t(8;21) AML

The clinical characteristics of patients are shown in Table 1. The median age was 50.3 years (range, 31–72 years) with 69.2% being males. Patients were followed-up for 3–112 months after initial presentation, with a median of 34.5 months. Of the 26 total t(8;21) AML cases, 15 (57.7%) relapsed.

Behenoyl cytosine arabinoside (BHAC)-DM-based, DNR/Ara-C and IDA/Ara-C regimens were used as induction therapies for 16, 7 and 3 cases, respectively. BHAC-DM-based regimen was a response-oriented individualized chemotherapy regimen using BHAC, dounorbicin (DNR) and 6-mercaptppurine (6-MP).[35–38] All patients obtained complete remission by induction therapies. For post-remission consolidation, BHAC-based and HDAC-based regimens were administered in 14 and 12 cases, respectively. As t(8;21) AML was considered to have favorable prognosis, SCT was not given in any of the cases during the first remission period.

Evaluation of post-remission course revealed a range of 1–64 months until first relapse (median 16 months) and CIR of 45.2% at 1 year and 58.6% at 6 years. We further analyzed the impact of post-remission therapy, WBC counts at initial examination, WBC index and CD56 expression on relapse. However, post-remission therapies, initial WBC counts, WBC index and CD56 expression were not significantly associated with relapse, and therefore, remain of unclear prognostic significance. Specifically, BHAC-based regimen had CIR of 31.9% at 1 year and 48.9% at 6 years, whereas HDAC-based regimen resulted in CIR of 58.3% at 1 year and 66.7% at 6 years ($P=0.384$). When initial WBC counts were equal to or greater than 20 000/μl, CIR was 60.0% at 1 year and 80.0% at 6 years. When initial WBC counts were less than 20 000/μl, CIR was 36.2% at 1 year and 48.4% at 6 years ($P=0.4343$). WBC index were equal to or greater than 20, CIR was 43.2% at 1 year and 54.1% at 6 years. When WBC index were less than 20, CIR was 33.3% at 1 year and 66.7% at 6 years ($P=0.7569$). CD56 expression less than 10% was associated with CIR of 25.0% at 1 year and 52.4% at 6 years, whereas CD56 expression greater or equal to 10% resulted in CIR of 57.7% at 1 years and 66.2% at 6 years ($P=0.083$).

Comparison of chromosome analysis at initial presentation and at relapse

There were 14/26 cases (53.8%), in which a chromosomal aberration other than t(8;21)(q22;q22) was observed at initial presentation (Table 2). Among these, the most frequent was the

Table 1 Clinical and molecular characteristics of t(8;21)AML patients

Characteristics	*Total*	*MB-PCR method*		
	t(8;21)AML n = 26	*Wild type n = 14*	*c-kit mutation(+) n = 12* (*D816V: 4, N822K: 8*)	P
Male/female	18/8	10/4	8/4	0.673
Age (years)				
Median (range)	50.3 (31–72)	50.2 (31–72)	50.4 (33–67)	1.000
<60	22	12	10	
≧60	4	2	2	
PS ≧2	1	1	0	
WBC (/μl)				
Median (range)	12858 (1400–42800)	12962 (1400–42800)	12745 (3900–29900)	0.928
<20 000	21	11	10	
≧20 000	5	3	2	
Hemoglobin (g/dl)				
Median (range)	7.48 (3.2–12.5)	6.73 (3.2–10.1)	8.36 (4.2–12.5)	0.148
≧10	21	13	8	
<10	5	1	4	
Platelet ($\times 10^4/\mu l$)				
Median (range)	2.26 (0.7–8.5)	2.43 (0.7–8.5)	2.08 (0.4–5.8)	0.654
% PB blast	49.8 (10.0–93.5)	58.7 (15.5–92.0)	48.2 (10.0–93.5)	0.770
Median (range)				
% BM blast				
Median (range)	64.5 (30.4–95.0)	65.3 (37.6–95.0)	63.5 (30.4–90.0)	0.832
WBC index				
Median (range)	70.6 (9.8–296.2)	67.4 (9.8–296.2)	74.8 (19.1–163.8)	0.792
<20	3	2	1	
≧20	23	12	11	
Extramedullary involvement	—	—	—	
CD56+				
<10%	13	9	4	0.238
≧10%	13	5	8	
Flt3 ITD				
Negative	24	13	11	
Positive	2	1	1	
Relapse rate (%)	57.7%	35.7%	83.3%	0.021
CIR				
Median	18 months	Not reached	11 months	0.014
%CIR at 1 year	45.2%	22.1%	72.5%	
%CIR at 6 years	58.6%	34.4%	81.7%	

Abbreviations: AML, acute myeloid leukemia; CIR, cumulative incidence of relapse; WBC, white blood cell.

loss of a sex chromosome, which was observed in 12 cases (46.2%) (−Y: 11 cases, −X: 1 case) (Table 2).

Of 15 relapsed cases, three were unacceptable for chromosomal analyses. Additional chromosomal aberrations were observed at relapse in 10/12 cases (83.3%), which was higher relative to the rate at initial presentation. New chromosomal aberrations were observed in 9/12 cases (75%). Furthermore, most of these cases were observed with two or more chromosomal aberrations in addition to t(8;21)(q22;q22). This suggests greater chromosomal instability at relapse compared with initial presentation. However, prognostic analyses did not show a significantly higher relapse rate (RR) for cases with additional chromosomal aberrations at initial examination ($P = 0.400$). Specifically, cases with additional chromosomal aberrations at initial examination had CIR of 30.4% at 1 year and 53.6% at 6 years, whereas those without additional chromosomal aberrations at initial examination had CIR of 63.3% at 1 year and 6 years.

c-kit mutations detected by direct sequence analysis

We screened for *c-kit* mutations using DS on 26 initial presentation cases and 13 relapsed cases with t(8;21) AML, other two relapsed cases were not available for analysis. In initial presentation subject samples, mutations were observed in 5/26 cases (19.2%; three cases of D816V and two cases of

Table 2 Chromosomal aberrations, direct sequence and MB-PCR analysis of *c-kit* gene at initial presentation and relapse

	%Blast	Chromosome analysis	Direct sequence	MB-PCR
At initial presentation				
1	67.4%	46,XY,t(8;21)(q22;q22)	D816V	D816V
2	57.8%	45,X-Y,t(8;21)(q22;q22)	D816V	D816V
3	66.8%	46,XX,t(8;21)(q22;q22)	D816V	D816V
4	46.5%	46,XY,t(8;21)(q22;q22)	Wild	D816V
5	55.0%	46,XY,t(8;21)(q22;q22)	Wild	N822K
6	67.6%	45,X-Y,t(8;21)(q22;q22)	N822K	N822K
7	48.8%	45,X-Y,t(8;21)(q22;q22)	N822K	N822K
8	48.4%	46,XX,t(8;21)(q22;q22)	Wild	N822K
9	30.4%	46,XY,t(8;21)(q22;q22)	M541 L	N822K
10	80.0%	46,XX,t(1;13;21;8)(q21;q14;q22;q22)	Wild	N822K
11	90.0%	46,XX,t(8;21)(q22;q22)	M541 L, K546 K, L862 L	N822K
12	32.4%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
13	69.2%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
14	49.6%	45,X-X,t(8;21)(q22;q22)	Wild	No signal
15	37.6%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
16	63.2%	46,XY,t(8;21)(q22;q22)	M541 L	No signal
17	90.0%	46,XY,t(8;21)(q22;q22)	Wild	N822K
18	62.8%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
19	54.8%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
20	95.0%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
21	44.2%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
22	70.0%	46,XX,t(8;21)(q22;q22)	Wild	No signal
23	95.0%	46,XX,t(8;21)(q22;q22)	Wild	No signal
24	79.8%	46,XY,t(8;21)(q22;q22)	Wild	No signal
25	62.6%	46,XX,t(8;13;21)(q22;q14;q22)	Wild	No signal
26	55.1%	45,X-Y,t(8;21)(q22;q22)	Wild	No signal
At relapse				
1	75.4%	46,XY,t(8;21)(q22;q22),t(1;2)(q42;q21) 46,XY,t(8;21;22)(q22;q22:q11)	D816V	D816V
2	82.4%	45,X-Y,t(8;21)(q22;q22),t(1;13)(p36;q14),del(11)(q23) 45,X-Y,t(8;21)(q22;q22),add(7)(q22)	D816V	D816V
3	42.0%	not acceptable	D816V	D816V
4	91.5%	45,X-Y,t(8;21)(q22;q22),t(1;22)(q21;q;q13) 45,X-Y,t(8;21)(q22;q22),add(2)(q33)	D816V	D816V
5	91.8%	45,X-Y,der(2)del(2)(p21)del(2)(q33),der(8)t(8;21)(q22;q22)t(8;14)(q22;q13),add(9)(p22),add(10)(q22),add(11)(q23),der(14)t(8;21)t(8;14),add(15)(q11),add(20)(q13),der(21)t(8;21)	D816V	D816V
6		Continuous first remission		
7	55.6%	45,X-Y,t(8;21)(q22;q22),add(2)(q21)	N822K	N822K
8	87.5%	46,XX,t(8;21)(q22;q22)	N822K	N822K
9	74.7%	46,XY,6q-,7q+,-11,t(8;21)(q22;q22),+mar	M541 L	No signal
10	42.2%	46,XX,t(1;13;21;8)(q21;q14;q22;q22)	wild	No signal
11	55.0%	46,XX,t(8;21)(q22;q22)	M541 L, K546 K, L862 L	No signal
12	30.2%	Not acceptable	Wild	No signal
13	70.2%	45,X-Y,t(8;21)(q22;q22),12q-	Not acceptable	Not acceptable
14	30.0%	45,X-X,t(6;10)(q15;p13),t(8;21)(q22;q22) 45,X-X,t(8;21)(q22;q22),add(16)(q24)	Wild	No signal
15	8.0%	Not acceptable	Not acceptable	Not acceptable
16	35.2%	45,X-Y,t(8;21)(q22;q22),t(11;12)(p13;q24.3) 45,X-Y,t(8;21)(q22;q22),t(9;12)(q22;q13),t(11;12)(p13;q24.3)	M541 L	No signal
17		Drop out		
18		Continuous first remission		
19		Continuous first remission		
20		Continuous first remission		
21		Continuous first remission		
22		Continuous first remission		
23		Continuous first remission		
24		Continuous first remission		
25		Continuous first remission		
26		Continuous first remission		

N822K). In relapsed subject samples, mutations were observed in 7/15 cases (46.7%; five cases of D816V and two cases of N822K, two cases were not acceptable). All *c-kit* mutations localized to the A-loop of exon 17. Intriguingly, of the relapsed cases with *c-kit* mutations, three cases (two cases of D816V and one case of N822K) were not detected *c-kit* mutation at initial presentation. Sequences of the detected c-kit mutations were D816V (GAT→GTC) and N822K (AAT→AAG). In addition to the above-mentioned mutations, we observed three cases of M541L (ATG→CTG; 11.5%), one case of K546K (AAA→AAG; 3.8%), and one case of L862L (CTG→CTC; 3.8%) as genetic polymorphisms (Table 2).

Establishment of a highly sensitive c-kit mutation detection method with MB-PCR

There were three cases, in which *c-kit* mutations were observed only at relapse using DS. For this reason, we surmised that minor clone of leukemic cells with *c-kit* mutations at initial presentation had resistance to treatment and became involved in relapse. To prove this hypothesis, we established a highly sensitive method for detecting these *c-kit* mutations called MB-PCR. The

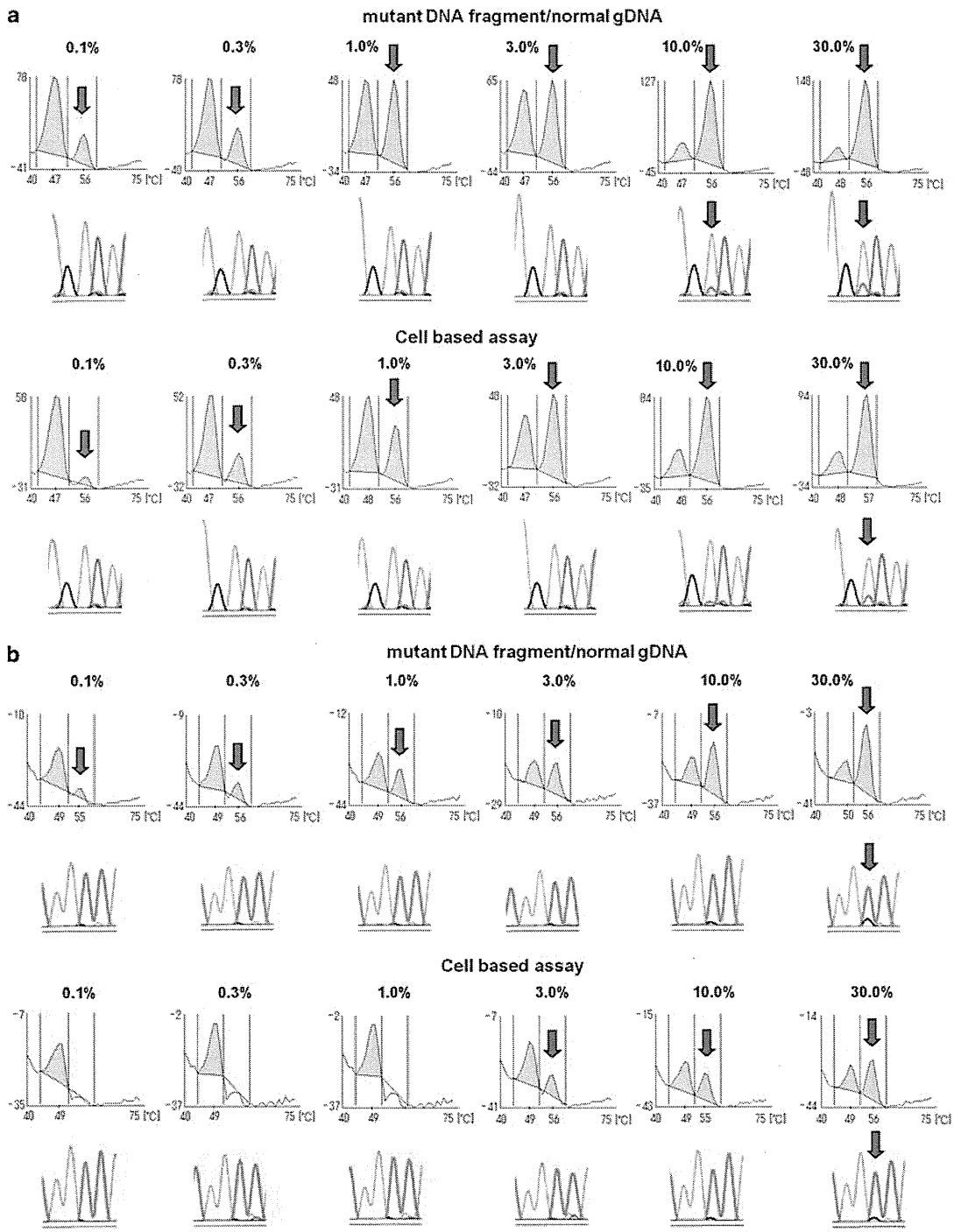

Figure 1 Comparison of detection sensitivities for c-kit exon 17 mutations. (**a**) Detection sensitivity for the D816V mutation. (**b**) Detection sensitivity for the N822K mutation. In (**a**) and (**b**), the upper panel shows the sensitivity analysis using a mixture of a mutated fragment and DNA from a normal subject. The lower panel shows sensitivity analysis using a mixed sample containing cells harboring mutations and normal cells. Sensitivity analysis was conducted using samples corresponding to 0.1, 0.3, 1, 3, 10 and 30% dilutions. The upper row in each panel shows results from MB-PCR. In contrast to wild-type *c-kit*, for which the complementary strand and QProbe separate at Tm values of 42–52 °C (D816V region) and 44–52 °C (N822K region), separation is observed at Tm values of 52–60 °C and 52–61 °C, respectively, for D816V and N822K mutations. The lower row of each panel shows results from the DS method. Arrows indicate waves of mutations.

sensitivity of detecting mutations using this method was tested by mixing cells containing D816V or N822K mutations with normal cells, which showed detection sensitivities of around 0.1% for D816V and 3.0% for N822K. Sensitivity analysis with a D816V- and N882K-containing fragment mixed with DNA from normal subjects produced a detection sensitivity of around 0.1% for both D816V and N822K. In contrast, the same sample subjected to the DS method resulted in a detection sensitivity of 10–30% for D816V and 30% for N822K. These results suggest that compared with the DS method, MB-PCR can detect mutations with much higher sensitivity (Figure 1).

Screening for c-kit mutations using highly sensitive mutation detection methods

When *c-kit* mutations of D816V and N822K were screened using MB-PCR, 12/26 of initial presentation samples were positive (46.2%; 4 cases of D816V and 8 cases of N822K). In 15 of relapse samples, two of them were not available for analyses, and seven of them were detected *c-kit* mutations (46.7%; five cases of D816V and two cases of N822K). In other words, MB-PCR offered a higher detection rate for *c-kit* mutations at initial presentation samples compared with DS.

MB-PCR was able to detect *c-kit* mutations at initial presentation or early stage for the three cases, in which *c-kit* mutations were observed only at relapse using DS. MB-PCR could detect *c-kit* mutations at initial presentation of case number 4 and 8, as well as at the first relapse of case number 5, for which *c-kit* mutations could not detected using DS (Figure 2).

Although less prevalent than the D816V and N822K mutations, there has been reports of small deletion-type mutation in the T417–D419 region of exon 8 in *c-kit* (i.e., del419, del418–419 and Thr417Ile/del418–419). In order to analyze this exon 8 mutation, we generated a QProbe (QProbe $^{T417-D419}$) directed at the wild-type sequence. However, the T417–D419 deletion could not be detected in first presentation and relapsed t(8;21) AML samples.

Clinical significance of c-kit D816V and N822K mutations

The clinical progression of the 11 cases with detected *c-kit* mutations are shown in Figure 3 (one case was not included due to a short observation period). Five cases with the D816V observed at initial presentation using the MB-PCR method all eventually relapsed, and at relapse D816V remained positive in all cases (Figure 3). For cases number 4 and 5 in particular, a low number of leukemic cells had D816V at initial presentation, which apparently became treatment resistant and amplified in subsequent relapse. Of the seven cases with N822K at initial presentation, six cases (86%) relapsed. Among these, however, N822K was observed in only two cases (33.3%) at relapse (Figure 3).

Significance of c-kit mutation as a prognostic factor in t(8;21) AML

Using DS and MB-PCR, we examined whether initial possession of a *c-kit* mutation has prognostic significance given the above MB-PCR results. No significant differences were found in clinical characteristics of case samples analyzed by both methods between those with or without *c-kit* mutations (Table 1 and Supplementary Table 1). By DS, there was a tendency for high RRs in the *c-kit* mutation (+) group, but this was not statistically significant (Figure 4a). Specifically, the *c-kit* mutation (+) group had a RR of 80.0% and CIR of 60.0% at 1 year and 80.0% at 6 years, while the *c-kit* mutation (−) group had a RR of 52.4% and CIR of 35.7% at 1 year and 52.8% at 6 years ($P=0.355$). However, with MB-PCR, the *c-kit* mutation (+) group had a statistically significant higher RR (Figure 4b). Specifically, the *c-kit* mutation (+) group had a RR of 83.3% and CIR of 72.5% at 1 year and 81.7% at 6 years, whereas the *c-kit* mutation (−) group had a RR of 35.7% and CIR of 22.1% at 1 year and 34.4% at 6 years ($P=0.014$).

Significance of c-kit mutation and Flt3ITD as a prognostic factor in t(8;21) AML

Flt3 ITD is observed in about 25% of all AMLs and is one of the gene mutations considered to be an adverse prognostic factor. However, among the 26 cases of t(8;21) AML examined, *Flt3 ITD* was observed in only 2/26 (7.7%) cases at initial presentation, which is a lower incidence relative to *c-kit* mutations. In one case, *Flt3 ITD* and *c-kit* mutations were observed simultaneously. All patients with *Flt3 ITD* demonstrated relapse after short remission period. Considering the *Flt3 ITD* (+) cases and/or MB-PCR *c-kit* mutation (+) cases together as the mutation (+) group, its relapse rate was shown to be significantly higher compared with those lacking both mutations (mutation (−) group: RR 30.8%, 1 year CIR 15.4%, 6 year CIR 34.2% versus mutation (+) group: RR 84.6%, 1 year CIR 74.8%, 6 year CIR 83.2%, $P=0.005$) (Figure 4c).

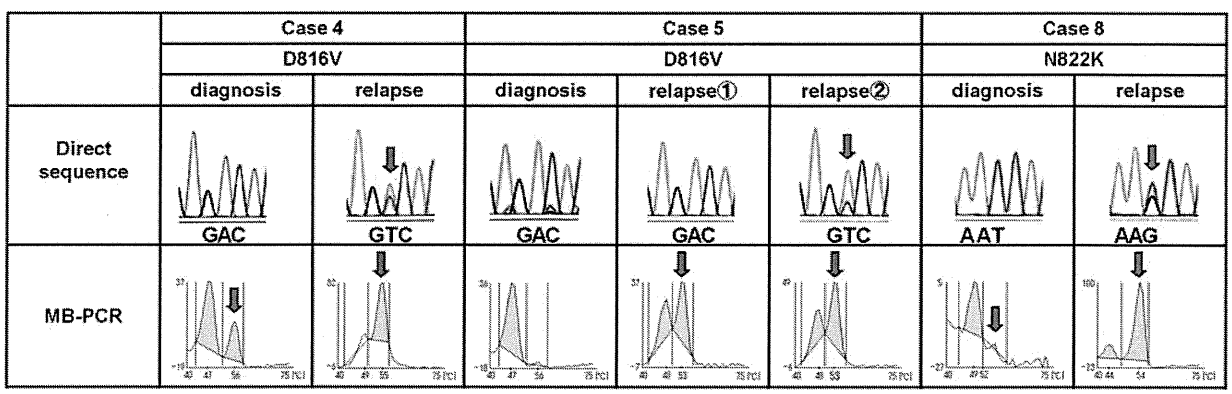

Figure 2 Detection of *c-kit* mutations with MB-PCR but not DS. The top and bottom panels, respectively, show results of the DS method and MB-PCR. For cases 4 and 8 at diagnosis and case 5 at first relapse, *c-kit* mutations were detected with MB-PCR but not with DS. Arrows indicate waves of mutations.

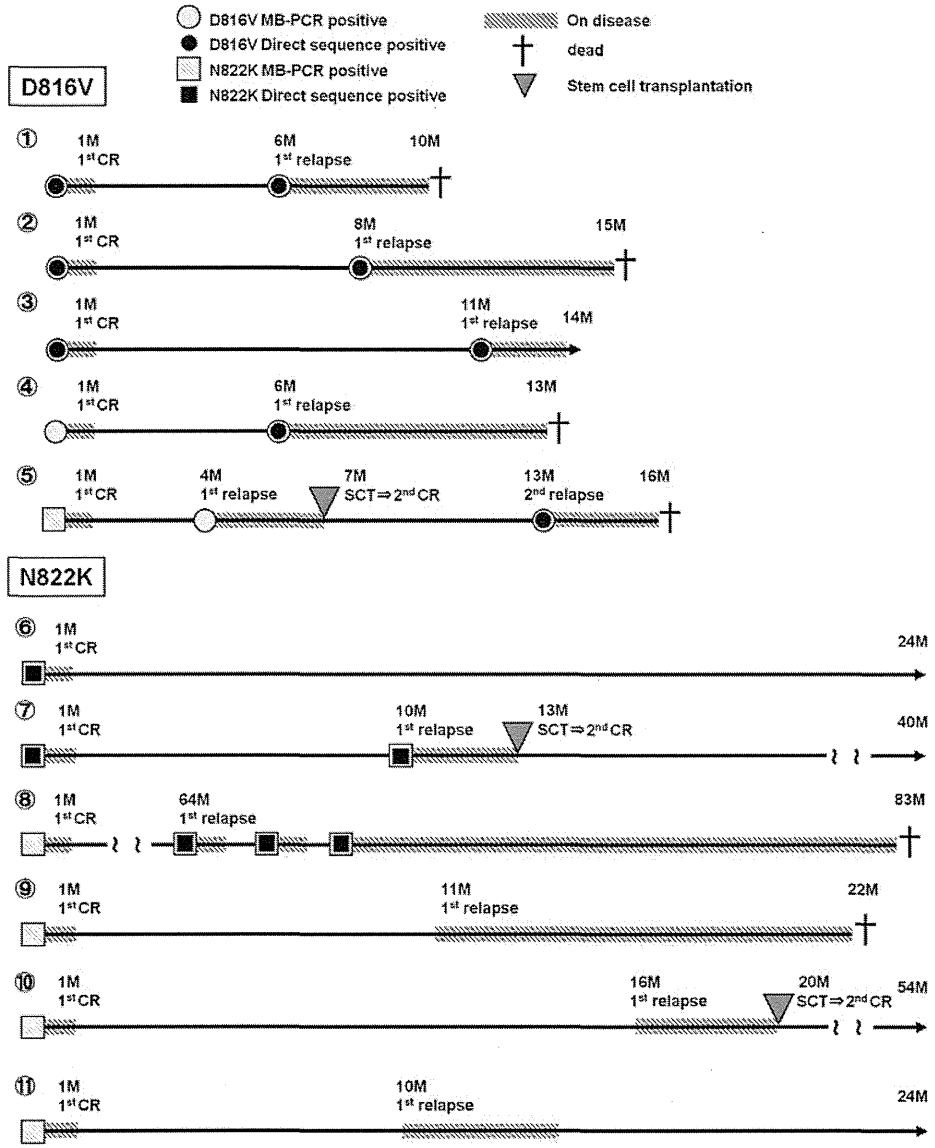

Figure 3 Clinical courses of patients with *c-kit* mutations D816V and N822K. The top panel shows five cases, in which the D816V mutation was observed; the bottom panel shows six cases, in which the N822K mutation was observed. For cases 4 and 5 of D816V and case 8 of N822K, mutations were detected with 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

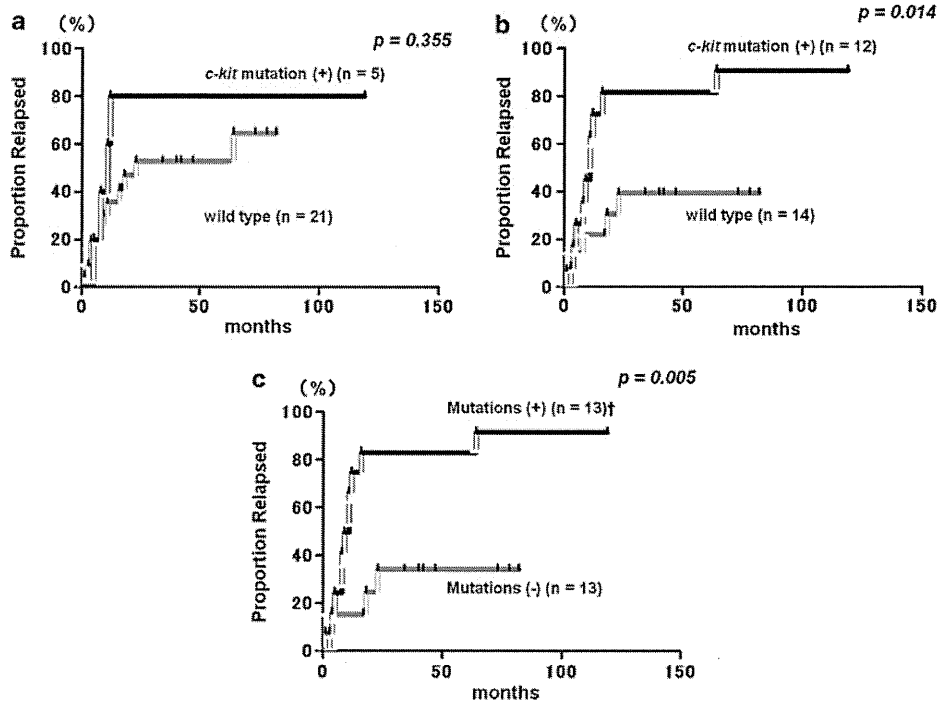

Figure 4 Relationship between presence of *c-kit* mutations and relapse. (**a**) Relationship between the presence of *c-kit* mutations and relapse with the DS method; (**b**) Relationship between the presence of *c-kit* mutations and relapse with the MB-PCR method; (**c**) Relationship between the presence 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

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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

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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 × 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.

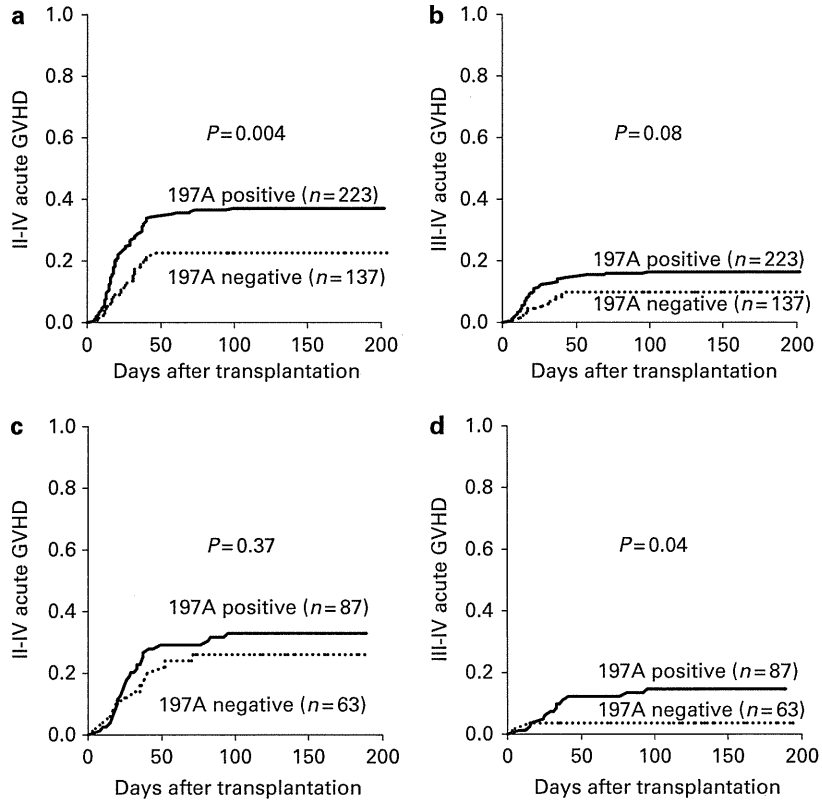

Figure 1 Estimated cumulative incidence curves of grades II–IV (**a**, **c**) and grades III–IV (**b**, **d**) acute GVHD according to the recipient IL-17 genotype in the discovery study (**a**, **b**) and the validation study (**c**, **d**).

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

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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

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

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