TAM.[19] Diagnosis of i-TAM is important because tapering of immunosuppressants may result in the resolution of intestinal symptoms and better survival.[19]

To clarify more detailed clinicopathological manifestations of i-TAM and its therapeutic strategy with a larger number of patients, we conducted a retrospective multicenter study in the Nagoya Blood and Marrow Transplantation Group.

Patients and methods

Patients

A total of 886 patients received allo-SCT in the Nagoya Blood and Marrow Transplantation Group from 1997 to 2006. Of these, 87 patients (10%) who received colonoscopic biopsy within 5 months after engraftment were evaluated. This retrospective study was approved by the ethics committee at Nagoya University Hospital.

Histopathological diagnosis

All specimens were fixed in formalin solution and embedded in paraffin. Sections were stained with hematoxylin–eosin. Immunostainings of CD34 (Novocatra, Newcastle, UK), CD8 (Novocatra) and CMV (DAKO, Copenhagen, Denmark) were performed using paraffin sections by Envision methods (DAKO) according to the manufacturer's instructions. Specimens were reviewed retrospectively by four expert pathologists in a blinded manner (MI, MF, RI and NH), and diagnosis was made according to the consensus among these pathologists.

Histopathological diagnosis of i-TAM was made by the presence of microangiopathy with ischemic (non-inflammatory) crypt loss (Figure 1). Microangiopathy was confirmed by hematoxylin–eosin staining and CD34 immunostaining. The clues for endothelial injury are swollen endothelial cells and denuded endothelial cells.[20] Ischemic changes followed by microangiopathy included individual non-inflammatory crypt degeneration with detachment and apoptosis of epithelial cells, wedge-shaped segmental injury and interstitial edema with hemorrhage or fragmented RBCs. Incomplete regeneration and residual neuroendocrine cells were also indicators of ischemic tissue damage. To identify residual neuroendocrine cells, either Grimelius staining or immunostaining for chromogranin A, CD56 or synaptophysin was used. Plt thrombi were not necessarily required for diagnosis because, unlike thrombotic thrombocytopenic purpura (TTP), systemic microthrombus formation has not been shown to play an essential role in TAM.[5,7,20]

Histopathological diagnosis of GVHD was based on apoptosis associated with intraepithelial lymphocytosis of cytotoxic T cells confirmed by CD8 immunostaining. Diagnosis of CMV colitis was based on intranuclear and intracytoplasmic amphophilic viral inclusion body and confirmed by positive CMV immunostaining.

Clinical data assessment

Retrospective chart reviews were performed to collect clinical data including (1) patient background (age, sex, disease, risk, donor type, graft source, HLA and ABO compatibilities, conditioning regimen, GVHD prophylaxis,

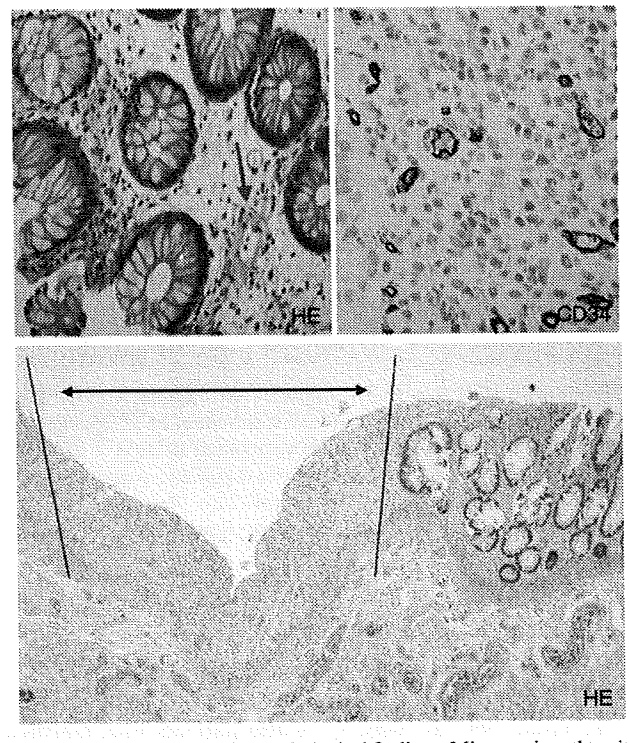

Figure 1 Colonoscopic histopathological findings. Microangiopathy with crypt loss in i-TAM (red arrow). CD34 immunostaining is helpful. Wedge-shaped segmental injury (black arrow). i-TAM, intestinal transplant-associated microangiopathy.

duration from transplantation to the onset of diarrhea and duration from the onset of diarrhea to biopsy); (2) doses of immunosuppressants before and after the onset of diarrhea; (3) laboratory findings (LDH, schistocytes, haptoglobin, direct and indirect Coombs tests, blood urea nitrogen and serum creatinine); (4) coexistent symptoms (abdominal pain, bloody diarrhea and neurological dysfunction); (5) skin or liver GVHD at biopsy; and (6) safety of colonoscopic biopsy (plt at biopsy and procedure-associated complications). Chronic GVHD was evaluated in patients surviving beyond day 100 and was classified as limited or extensive according to the Seattle criteria.[21] Diagnosis of systemic TAM was made by BMT CTN criteria and modified EBMT criteria (more than two schistocytes per high-power field on peripheral smear, thrombocytopenia, elevated LDH and decreased haptoglobin).

Treatment for diarrhea was determined by the physician in charge at the time. Accordingly, patients could be divided into four groups by the way of immunosuppressant use (steroid, CYA and tacrolimus) 1 week before and after the onset of diarrhea: intensified–intensified (I–I) group, intensified–not intensified (I–N) group, not intensified–intensified (N–I) group and not intensified–not intensified (N–N) group. 'Intensified' was defined by increments of any immunosuppressant, and 'not intensified' was defined by maintenance or tapering of all immunosuppressants.

Statistical analysis

The aim of this study was to evaluate more detailed clinicopathological manifestations of i-TAM and safety

and effectiveness of 'not intensified' strategy for i-TAM. OS was calculated using the Kaplan–Meier method. Cumulative incidences of non-relapse mortality (NRM) and chronic GVHD were estimated by Gray's method, with relapse and deaths not related to GVHD as competing risks, respectively. Univariate and multivariate analyses were performed using the Cox proportional hazard regression model. Analysis was performed using STATA (StataCorp. 2007; Stata Statistical Software: Release 10.0. Special Edition; Stata Corporation, TX, USA). Data analyses were completed as of March 2007.

Results

Patient characteristics

Patient characteristics are summarized in Table 1. Median age was 41 years. More male patients were included. Diseases were heterogenous hematological malignancies. Disease risk was standard in 33 patients and high in 54 patients; standard risk included acute leukemia and lymphoma in the first and second remission, CML in chronic phase and myelodysplastic syndrome in refractory anemia with or without ringed sideroblasts, whereas high risk included others. Donor was related in 32 patients and unrelated in 55 patients. Graft source was BM in 69 patients, PBSCs in 13 patients and cord blood in 5 patients. HLA was genotypically matched at A, B and DRB1 loci in 45 patients and mismatched in 42 patients (1 Ag mismatch at class I locus ($n=13$), 1 serological Ag mismatch at DRB1 locus ($n=12$), 1 genotypical Ag mismatch at DRB1 locus ($n=9$) and 2 Ag mismatches ($n=8$)). ABO was matched in 41 patients and mismatched in 46 patients. Conditioning regimen was myeloablative in 79 patients and non-myeloablative in 8 patients. Tacrolimus was used as GVHD prophylaxis in 59 patients and CYA in 28 patients. Median duration from transplantation to the onset of diarrhea was 32 days (range: 9–130 days) and that from the onset of diarrhea to biopsy was 12 days (range: 0–74 days).

Safety of colonoscopic biopsy

A total colonoscopic exam was performed in all patients. No procedure-associated complications developed with plt transfusion for 30 patients, although plt at biopsy was less than 2×10^{10}/l in 12 patients.

Histopathological diagnosis and findings

Histopathological diagnosis in each patient is summarized in Figure 2. i-TAM was confirmed in 80 patients (92%). GVHD was confirmed in 26 patients (30%) and was coexistent with i-TAM in 23 patients. The incidence of histopathological GVHD was lower among patients without steroid at biopsy than among those with steroid at biopsy, although the difference was not statistically significant (24 vs 38%; $P=0.24$). CMV was confirmed in 17 patients (20%). Findings were nonspecific in two patients.

Histopathological findings of i-TAM included microangiopathy with crypt loss (100%), interstitial edema with hemorrhage with or without fragmented RBCs (39%), crypt degeneration with detachment and/or apoptotic epithelial cells (93%), residual neuroendocrine cells (43%) and plt thrombi (30%) (Table 2). The affected sites of the

Table 1 Patient characteristics

Number of patients	87
Median age in years (range)	41 (18–61)
Sex (M/F)	65/22
Disease	
AML	20
ALL	18
CML	18
MDS	17
ML	11
ATL	3
Disease risk	
Standard	33
High	54
Donor	
Related	32
Unrelated	55
Stem cell source	
BM	69
PBSC	13
Cord blood	5
HLA disparity (A, B, DRB1 loci)	
Match	45
Mismatch	42
ABO compatibility	
Match	41
Major mismatch	16
Minor mismatch	19
Major–minor mismatch	11
Conditioning	
Myeloablative	79
Non-myeloablative	8
GVHD prophylaxis	
Tacrolimus + MTX	59
CYA + MTX	27
CYA	1
Median duration from transplant to the onset of diarrhea (range)	32 (9–130)
Median duration from the onset of diarrhea to biopsy (range)	12 (0–74)

Abbreviations: ATL = adult T-cell leukemia/lymphoma; MDS = myelodysplastic syndrome; ML = malignant lymphoma.

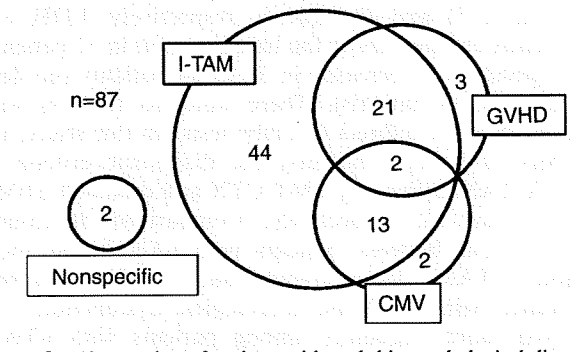

Figure 2 The number of patients with each histopathological diagnosis. i-TAM, intestinal transplant-associated microangiopathy.

Table 2 Histopathological findings of i-TAM

	Incidence (%)
Microangiopathy with crypt loss	80 (100)
Interstitial edema with hemorrhage with or without fragmented RBCs	31 (39)
Crypt degeneration with detachment and/or apoptotic epithelial cells	74 (93)
Residual neuroendocrine cells	34 (43)
Plt thrombi	24 (30)

Abbreviation: i-TAM = intestinal transplant-associated microangiopathy.

Table 3 Affected sites of intestine

	Incidence (%)
Sites	
Terminal ileum	90
Ascending colon	79
Transverse colon	83
Descending colon	77
Sigmoid colon	73
Rectum	73
Affected pattern	
Regional/diffuse	33%/67%

intestine were terminal ileum (90%), ascending colon (79%), transverse colon (83%), descending colon (77%), sigmoid colon (73%) and rectum (73%). The affected pattern was regional in 33% of patients and diffuse in 67% of the patients (Table 3).

Clinical manifestations

The median maximal amount of diarrhea was 21/day (range: 130–5600 ml/day). Skin and/or liver GVHD before the onset of diarrhea was observed in 50 patients. Abdominal pain and bloody diarrhea at biopsy were observed in 70 and 26 patients, respectively. Veno-occlusive disease (VOD) was observed in six patients. Renal or neurological dysfunction was observed in 13 patients. Prolonged or progressive thrombocytopenia (plt count less than 5×10^{10}/l) and schistocytes on peripheral smear were observed in 51 and 40 patients, respectively. LDH was persistently elevated above the baseline value in 57 patients. Haptoglobin was decreased in 33 of 64 patients (no data available in 23 patients). There were no patients with positive direct or indirect Coombs tests. In this study, the numbers of patients fulfilling the diagnostic criteria of systemic TAM defined by BMT CTN and modified EBMT were 10 and 12, respectively. Comparison of clinical manifestations between patients with i-TAM and those without i-TAM demonstrated that massive diarrhea, abdominal pain, VOD and neurological dysfunction were observed more frequently among patients with i-TAM (Table 4).

Clinical outcome

OS after biopsy among 80 patients with i-TAM was 30% at 2 years. Diarrhea resolved in 51 patients, but NRM was as high as 33% among them and the relapse rate of the underlying disease was 27%. The causes of NRM in these patients were infection ($n=8$), systemic TAM ($n=3$), chronic GVHD ($n=3$), bronchiolitis obliterans-organizing pneumonia ($n=1$), VOD ($n=1$) and late graft failure ($n=1$). NRM increased significantly to 72% in patients without resolution of diarrhea ($P=0.001$). Their relapse rate of the underlying disease was 21%. One patient is alive with persistent diarrhea at 2 years and 5 months after transplantation. The causes of NRM in these patients were i-TAM ($n=12$), infection ($n=4$), interstitial pneumonia ($n=3$), VOD ($n=1$) and chronic GVHD ($n=1$). The cumulative incidence of chronic GVHD was 58% in 71 evaluable patients. Of 36 patients with chronic GVHD, 4 had the limited and 32 the extensive type.

Table 4 Comparison of clinical manifestations between patients with and without i-TAM

Clinical manifestations	*With i-TAM (n = 80)*	*Without i-TAM (n = 7)*
Median maximal amount of diarrhea in ml/day (range)	2000 (130–5640)	800 (640–1900)
Abdominal pain	66 (83%)	4 (57%)
Bloody diarrhea at biopsy	24 (30%)	2 (29%)
Skin eruption	53 (66%)	5 (71%)
Veno-occlusive disease	6 (7.5%)	0 (0%)
Renal dysfunction	9 (11%)	1 (14%)
Neurological dysfunction	7 (8.8%)	0 (0%)
Thrombocytopenia	46 (58%)	5 (71%)
Schistocyte on peripheral smear	37 (46%)	3 (43%)
Elevated LDH	52 (65%)	5 (71%)
Decreased haptoglobin	30/58 (52%)	3/6 (50%)
Systemic TAM by BMT CTN criteria	9 (11%)	1 (14%)
Systemic TAM by modified EBMT criteria	11 (14%)	1 (14%)

Abbreviations: BMT CTN = Blood and Marrow Transplant Clinical Trials Network; EBMT = European Group for Blood and Marrow Transplantation; LDH = lactate dehydrogenase; TAM = transplant-associated microangiopathy.

The impact of treatment on clinical outcome

Among 80 patients with i-TAM, 33 received steroid therapy at biopsy. Before the onset of diarrhea, immunosuppressants were intensified in 30 patients and maintained in 50 patients. After the onset of diarrhea, immunosuppressants were intensified in 23 patients and maintained or tapered in 57 patients. Accordingly, I–I group included 6 patients, I–N group 24 patients, N–I group 17 patients and N–N group 33 patients. Resolution rates of diarrhea in each group were 50, 67, 65 and 64%, respectively ($P=0.91$). Rates of NRM at 4 years in each group were 100, 79, 52 and 25%, respectively ($P=0.0001$; Figure 3). Subgroup analyses of the patients with i-TAM alone ($n=44$) and those with i-TAM and histopathological GVHD ($n=21$) also demonstrated similar results among the four groups ($P=0.0019$ and $P=0.0005$, respectively). After the onset of diarrhea, deterioration of skin or liver GVHD was observed in only 2 of 57 patients (3.5%) in the 'not intensified' group. After the onset of diarrhea, the incidence of chronic GVHD was higher in 'intensified' group than in

'not intensified' group, although the difference was not statistically significant (83 vs 49%; $P=0.14$).

Prognostic factors affecting NRM

In univariate analyses, significant factors affecting NRM were bloody diarrhea at biopsy (hazard ratio (HR) = 2.3; confidence interval (CI), 1.2–4.4; $P=0.011$) and intensified immunosuppression before diarrhea (HR = 2.6; CI, 1.3–4.9; $P=0.004$) (Table 5). GVHD prophylaxis with tacrolimus (HR = 2.0; CI, 0.95–4.3; $P=0.067$), absence of histopathological GVHD (HR = 2.1; CI, 0.94–4.5; $P=0.071$) and intensified immunosuppression after diarrhea (HR = 1.8; CI, 0.90–3.5; $P=0.093$) were factors with marginal significance. Other factors listed in Table 5 were not prognostic. In multivariate analyses, GVHD prophylaxis with tacrolimus (HR = 2.3; CI, 1.0–5.3; $P=0.041$), absence of histopathological GVHD (HR = 2.8; CI, 1.2–6.2; $P=0.014$), intensified immunosuppression before diarrhea (HR = 3.1; CI, 1.6–6.3; $P=0.001$) and intensified immunosuppression after diarrhea (HR = 2.9; CI, 1.4–6.1; $P=0.004$) were significant prognostic factors for NRM. Adjusted by donor type, GVHD prophylaxis with tacrolimus was not a significant factor (HR = 2.3; CI, 0.97–5.5; $P=0.060$). Even adjusted by the use of steroid at biopsy, the absence of histopathological GVHD remained a significant factor (HR 2.9; CI 1.3–6.4; $P=0.011$) (Table 5).

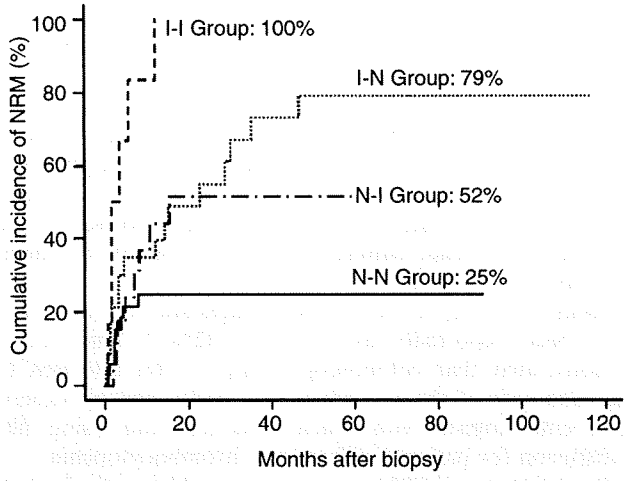

Figure 3 Cumulative incidence of non-relapse mortality according to the way of immunosuppressant use. NRM, non-relapse mortality; I–I, intensified–intensified; I–N, intensified–not intensified; N–I, not intensified–intensified; N–N, not intensified–not intensified.

Discussion

This study demonstrated that i-TAM was a major histopathological diagnosis in patients with severe diarrhea after allo-HSCT, comprising 92% of the patients. i-TAM was a major cause of NRM (57%) in patients without resolution of diarrhea. Massive refractory diarrhea (particularly more than 21/day) was a manifestation of i-TAM. Abdominal pain similar to ischemic colitis was a frequent symptom of i-TAM as we have reported earlier.[19] VOD and neurological dysfunction were observed only among patients with i-TAM, suggesting their relation to this complication.

Sternberg *et al.*[20] described in the pathology textbook that thrombi might or might not be seen in thrombotic microangiopathies and there was thickening and/or thrombosis of arterioles. Recently, Ruutu *et al.*[5] described that the Working Group wanted to avoid the word thrombotic, as systemic microthrombus formation had not been shown to play an essential role in TAM, contrary to *de novo* TTP. Although some investigators believe that plt thrombi are the main features of TAM, the main feature of TAM is microangiopathy. In addition, sampling errors are possible in detecting plt thrombi. Therefore, thrombi are not necessary for the diagnosis of i-TAM, and we use the term TAM instead of TMA (thrombotic microangiopathy). It is interesting to note that the ileum was affected most frequently. Intestinal ischemia was reported as a significant early postoperative complication after renal transplantation, and the most common sites were the terminal ileum

Table 5 Factors affecting non-relapse mortality

Variable	*Adverse factor*	*Univariate*		*Multivariate*[a]	
		Hazard ratio (CI)	P-*value*	*Hazard ratio (CI)*	P-*value*
Disease risk	High	1.3 (0.71–2.5)	0.36		
Patient age	Above 40 years	1.0 (0.53–1.9)	0.98		
Donor	Unrelated	1.6 (0.78–3.2)	0.20		
HLA	Mismatch	1.4 (0.73–2.6)	0.32		
ABO	Mismatch	1.4 (0.71–2.6)	0.35		
GVHD prophylaxis	Tacrolimus	2.0 (0.95–4.3)	0.067	2.3 (1.0–5.3)	0.041[b]
Diarrhea at biopsy	Bloody	2.3 (1.2–4.4)	0.011		
Skin/liver acute GVHD	Coexistent	1.2 (0.62–2.3)	0.58		
Histopathological GVHD	None	2.1 (0.94–4.5)	0.071	2.8 (1.2–6.2)	0.014[c]
Histopathological CMV	Positive	1.6 (0.76–3.3)	0.22		
IS before diarrhea	Intensified	2.6 (1.3–4.9)	0.004	3.1 (1.6–6.3)	0.001
IS after diarrhea	Intensified	1.8 (0.90–3.5)	0.093	2.9 (1.4–6.1)	0.004

Abbreviations: CI = confidence interval; IS = immunosuppressants.
[a]Final model.
[b]Adjusted by donor type, this factor was not significant (hazard ratio 2.3; CI 0.97–5.5; $P=0.060$).
[c]Adjusted by the use of steroid at biopsy, this factor remained significant (hazard ratio 2.9; CI 1.3–6.4; $P=0.011$).

and ascending colon.[22] Its mechanism is multifactorial, but the use of combination immunosuppressive agents in the early period is one of the causes.[22] Ischemic colitis with a preference for the terminal ileum might be a notable complication associated with transplantation.

Most patients were clinically diagnosed with intestinal GVHD, but apoptosis with intraepithelial lymphocytosis, which is a histopathological evidence of GVHD, was confirmed in only 30% of the patients. Although the use of steroid at biopsy may contribute to an underestimation of histopathological GVHD, such an underestimation is unlikely because histopathological GVHD was confirmed less frequently among patients without steroid at biopsy than among patients with steroid at biopsy. Interestingly, the absence of histopathological GVHD resulted in a higher NRM. Even adjusted with the use of steroid at biopsy, the absence of histopathological GVHD still remained a significant factor. This suggests that histopathological GVHD can be improved with immunosuppressive treatment, whereas i-TAM is refractory to treatment because microangiopathy is unlikely to improve with immunosuppressants.

The central nervous system and kidney are involved independently by TAM, which is known as nephrotoxicity or neurotoxicity with microangiopathic hemolytic anemia.[2] Likewise, the intestine was involved independently despite the absence of systemic TAM. However, the proposed criteria for systemic TAM by two groups[4,5] were not sufficient to make a diagnosis of i-TAM, because less than 15% of the patients fulfilled these criteria. The low matching rates of i-TAM and systemic TAM defined by the international panels' criteria suggest that systemic symptoms such as renal or neurological dysfunction, thrombocytopenia, schistocyte, elevated LDH and decreased haptoglobin have limitations for the diagnosis of i-TAM, and colonoscopic biopsy is the most reliable diagnostic method. i-TAM is a novel concept that cannot be diagnosed by the current scheme of transplant medicine. Other biomarkers of i-TAM are to be sought in the future.

The vascular endothelial damage of calcineurin inhibitors and steroid is one of the most important factors for the onset and deterioration of systemic TAM.[15] Several reports have documented an increased incidence of systemic TAM with sirolimus in combination with other calcineurin inhibitors.[23,24] Systemic TAM resolved with discontinuation of calcineurin inhibitors in most cases. In addition, we recently established a rat model of intestinal microangiopathy induced by tacrolimus alone.[25] The pathology was consistent with human i-TAM after allo-HSCT, and rats recovered from microangiopathy after withdrawal of tacrolimus. Therefore, we hypothesized that 'not intensified' strategy for i-TAM would minimize drug-induced endothelial damage and improve outcome, and divided patients into four groups according to the way of immunosuppressant use. The fact that resolution of diarrhea was achieved without intensification of immunosuppressants is of considerable interest and suggests that many cases of refractory colitis after allo-HSCT are due to i-TAM rather than GVHD. Figure 2 does not mean the overlap of entities but instead the overlap of histopathological diagnosis. We believe that i-TAM has a wide spectrum from i-TAM only to i-TAM and GVHD/CMV. Even limited to the 21 patients with histopathological GVHD, the 'not intensified' strategy improved outcome. If these patients had only GVHD, the results were rather paradoxical. Their therapeutic response suggested a need for a uniform treatment strategy.

Vascular endothelial cells may be targets in acute GVHD, but they were reported to escape an attack by cytotoxic T cells due to their impaired capacity to present antigenic peptides.[26] A French group reported that vascular endothelial cells in intestine could be targets of alloimmune reactions,[27] but they did not recognize that T cells were almost absent histopathologically when diarrhea became refractory. Their histopathological features were consistent with i-TAM. Even if therapy for acute GVHD was intensified for their cases, the outcome would not be improved. When a diagnosis of i-TAM is made, avoiding intensification of immunosuppressants until the resolution of i-TAM will improve diarrhea and the transplant outcome. Avoiding intensification may cause deterioration of GVHD, but only 3.5% of the patients experienced deterioration of GVHD in this study. Therefore, the 'not intensified' strategy was feasible.

On the other hand, there is a therapeutic dilemma as i-TAM is coexistent with GVHD in 30% of the patients. For these patients, GVHD should be treated promptly, but much attention should be paid to deterioration of i-TAM. Novel ways of immunosuppression, which do not cause vascular endothelial damage, are to be sought for these patients in the future.

Rates of complications after diagnostic colonoscopy have been reportedly as low as 0.02%.[28] This study demonstrated that colonoscopic biopsy after allo-HSCT was also safe, if the procedure was performed by expert gastroenterologists with much attention and using plt transfusion for patients with severe thrombocytopenia.

In summary, i-TAM was a major histopathological finding among patients with severe diarrhea. Massive refractory diarrhea and abdominal pain were manifestations of this complication. For patients with i-TAM, avoiding intensification of immunosuppression, which damages vascular endothelium until the resolution of i-TAM, may improve transplant outcome. Prospective trials are warranted to confirm the results, given the limitations of our analysis that include the various timing of biopsy and a possible selection bias for treatment. Our results will provide clues for the etiology and treatment of refractory diarrhea after allo-HSCT.

References

1 Zeigler ZR, Shadduck RK, Nemunaitis J, Andrews DF, Rosenfeld CS. Bone marrow transplant-associated thrombotic microangiopathy: a case series. *Bone Marrow Transplant* 1995; **15**: 247–253.
2 Pettitt AR, Clark RE. Thrombotic microangiopathy following bone marrow transplantation. *Bone Marrow Transplant* 1994; **14**: 495–504.
3 Ruutu T, Hermans J, Niederwieser D, Gratwohl A, Kiehl M, Volin L *et al.* Thrombotic thrombocytopenic purpura after

allogeneic stem cell transplantation: a survey of the European Group for Blood and Marrow Transplantation (EBMT). *Br J Haematol* 2002; **118**: 1112–1119.
4 Ho VT, Cutler C, Carter S, Martin P, Adams R, Horowitz M *et al.* Blood and marrow transplant clinical trials network toxicity committee consensus summary: thrombotic microangiopathy after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 571–575.
5 Ruutu T, Barosi G, Benjamin RJ, Clark RE, George JN, Gratwohl A *et al.* Diagnostic criteria for hematopoietic stem cell transplant-associated microangiopathy: results of a consensus process by an International Working Group. *Haematologica* 2007; **92**: 95–100.
6 Holler E, Kolb HJ, Hiller E, Mraz W, Lehmacher W, Gleixner B *et al.* Microangiopathy in patients on cyclosporine prophylaxis who developed acute graft-versus-host disease after HLA-identical bone marrow transplantation. *Blood* 1989; **73**: 2018–2024.
7 George JN, Li X, McMinn JR, Terrell DR, Vesely SK, Selby GB. Thrombotic thrombocytopenic purpura-hemolytic uremic syndrome following allogeneic HPC transplantation: a diagnostic dilemma. *Transfusion* 2004; **44**: 294–304.
8 Hahn T, Alam AR, Lawrence D, Ford L, Baer MR, Bambach B *et al.* Thrombotic microangiopathy after allogeneic blood and marrow transplantation is associated with dose-intensive myeloablative conditioning regimens, unrelated donor, and methylprednisolone T-cell depletion. *Transplantation* 2004; **78**: 1515–1522.
9 Shimoni A, Yeshurun M, Hardan I, Avigdor A, Ben-Bassat I, Nagler A. Thrombotic microangiopathy after allogeneic stem cell transplantation in the era of reduced-intensity conditioning: the incidence is not reduced. *Biol Blood Marrow Transplant* 2004; **10**: 484–493.
10 Kojouri K, George JN. Thrombotic microangiopathy following allogeneic hematopoietic stem cell transplantation. *Curr Opin Oncol* 2007; **19**: 148–154.
11 Nakamae H, Yamane T, Hasegawa T, Nakamae M, Terada Y, Hagihara K *et al.* Risk factor analysis for thrombotic microangiopathy after reduced-intensity or myeloablative allogeneic hematopoietic stem cell transplantation. *Am J Hematol* 2006; **81**: 525–531.
12 Uderzo C, Bonanomi S, Busca A, Renoldi M, Ferrari P, Iacobelli M *et al.* Risk factors and severe outcome in thrombotic microangiopathy after allogeneic hematopoietic stem cell transplantation. *Transplantation* 2006; **82**: 638–644.
13 Oran B, Donato M, Aleman A, Hosing C, Korbling M, Detry MA *et al.* Transplant-associated microangiopathy in patients receiving tacrolimus following allogeneic stem cell transplantation: risk factors and response to treatment. *Biol Blood Marrow Transplant* 2007; **13**: 469–477.
14 Martinez MT, Bucher C, Stussi G, Heim D, Buser A, Tsakiris DA *et al.* Transplant-associated microangiopathy (TAM) in recipients of allogeneic hematopoietic stem cell transplants. *Bone Marrow Transplant* 2005; **36**: 993–1000.
15 Lewis GD, Campbell WB, Johnson AR. Inhibition of prostaglandin synthesis by glucocorticoids in human endothelial cells. *Endocrinology* 1986; **119**: 62–69.
16 Cox GJ, Matsui SM, Lo RS, Hinds M, Bowden RA, Hackman RC *et al.* Etiology and outcome of diarrhea after marrow transplantation: a prospective study. *Gastroenterology* 1994; **107**: 1398–1407.
17 Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR *et al.* A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. *Blood* 1990; **76**: 1464–1472.
18 Deeg HJ. How I treat refractory acute GVHD. *Blood* 2007; **109**: 4119–4126.
19 Nishida T, Hamaguchi M, Hirabayashi N, Haneda M, Terakura S, Atsuta Y *et al.* Intestinal thrombotic microangiopathy after allogeneic bone marrow transplantation: a clinical imitator of acute enteric graft-versus-host disease. *Bone Marrow Transplant* 2004; **33**: 1143–1150.
20 Nadasdy T, Silva FG. Adult renal disease. In: Sternberg SS (ed). *Diagnostic Surgical Pathology*, 3rd edn, Lippincott Williams & Wilkins: Philadelphia, PA, 1999, pp 1736–1738.
21 Sullivan KM, Agura E, Anasetti C, Appelbaum F, Badger C, Bearman S *et al.* Chronic graft-versus-host disease and other late complications of bone marrow transplantation. *Semin Hematol* 1991; **28**: 250–259.
22 Dee SL, Butt K, Ramaswamy G. Intestinal ischemia. *Arch Pathol Lab Med* 2002; **126**: 1201–1204.
23 Couriel DR, Saliba R, Escalon MP, Hsu Y, Ghosh S, Ippoliti C *et al.* Sirolimus in combination with tacrolimus and corticosteroids for the treatment of resistant chronic graft-versus-host disease. *Br J Haematol* 2005; **130**: 409–417.
24 Cutler C, Henry NL, Magee C, Li S, Kim HT, Alyea E *et al.* Sirolimus and thrombotic microangiopathy after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 551–557.
25 Fujino M, Kim Y, Ito M. Intestinal thrombotic microangiopathy induced by FK506 in rats. *Bone Marrow Transplant* 2007; **39**: 367–372.
26 Kummer M, Lev A, Reiter Y, Biedermann BC. Vascular endothelial cells have impaired capacity to present immunodominant, antigenic peptides: a mechanism of cell type-specific immune escape. *J Immunol* 2005; **174**: 1947–1953.
27 Ertault-Daneshpouy M, Leboeuf C, Lemann M, Bouhidel F, Ades L, Gluckman E *et al.* Pericapillary hemorrhage as criterion of severe human digestive graft-versus-host disease. *Blood* 2004; **103**: 4681–4684.
28 Sieg A, Hachmoeller-Eisenbach U, Eisenbach T. Prospective evaluation of complications in outpatient GI endoscopy: a survey among German gastroenterologists. *Gastrointest Endosc* 2001; **53**: 620–627.

HapMap scanning of novel human minor histocompatibility antigens

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Minor histocompatibility antigens (mHags) are molecular targets of alloimmunity associated with hematopoietic stem cell transplantation (HSCT) and involved in graft-versus-host disease, but they also have beneficial antitumor activity. mHags are typically defined by host SNPs that are not shared by the donor and are immunologically recognized by cytotoxic T cells isolated from post-HSCT patients. However, the number of molecularly identified mHags is still too small to allow prospective studies of their clinical importance in transplantation medicine, mostly due to the lack of an efficient method for isolation. Here we show that when combined with conventional immunologic assays, the large data set from the International HapMap Project can be directly used for genetic mapping of novel mHags. Based on the immunologically determined mHag status in HapMap panels, a target mHag locus can be uniquely mapped through whole genome association scanning taking advantage of the unprecedented resolution and power obtained with more than 3 000 000 markers. The feasibility of our approach could be supported by extensive simulations and further confirmed by actually isolating 2 novel mHags as well as 1 previously identified example. The HapMap data set represents an invaluable resource for investigating human variation, with obvious applications in genetic mapping of clinically relevant human traits. (Blood. 2009;113:5041-5048)

Introduction

The antitumor activity of allogeneic hematopoietic stem cell transplantation (HSCT), which is a curative treatment for many patients with hematologic malignancies, is mediated in part by immune responses that are elicited as a consequence of incompatibility in genetic polymorphisms between the donor and the recipient.[1,2] Analysis of patients treated for posttransplantation relapse with donor lymphocytes has shown tumor regression to be correlated with expansion of cytotoxic T lymphocytes (CTLs) specific for hematopoiesis-restricted minor histocompatibility antigens (mHags).[3,4] mHags are peptides, presented by major histocompatibility complex (MHC) molecules, derived from intracellular proteins that differ between donor and recipient due mostly to single nucleotide polymorphisms (SNPs) or copy number variations (CNVs).[1,2,5] Identification and characterization of mHags that are specifically expressed in hematopoietic but not in other normal tissues could contribute to graft-versus-leukemia/lymphoma (GVL) effects, while minimizing unfavorable graft-versus-host disease, one of the most serious complications of allo-HSCT.[1,2] Unfortunately, however, efforts to prospectively target mHags to invoke T cell–mediated selective GVL effects have been hampered by the scarcity of eligible mHags, largely due to the lack of efficient methods for mapping the relevant genetic loci. Several methods have been developed to identify mHags, including peptide elution from MHC,[6,7] cDNA expression cloning,[8,9] and linkage analysis.[3,10] We have recently reported a novel genetic method that combines whole genome association scanning with conventional chromium release cytotoxicity assays (CRAs). With this approach the genetic loci of the mHag gene recognized by a given CTL clone can be precisely identified using SNP array analysis of pooled DNA generated from immortalized lymphoblastoid cell lines (LCLs) that are immunophenotyped into $mHag^+$ and $mHag^-$ groups by CRA.[11] The mapping resolution has now been improved from several Mb for conventional linkage analysis to an average haplotype block size of less than 100 kb,[12] usually containing a handful of candidate genes. Nevertheless, it still requires laborious DNA pooling and scanning of SNP arrays with professional expertise for individual

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CTLs.[11] To circumvent these drawbacks, we have sought to take advantage of publicly available HapMap resources. Here, we describe a powerful approach for rapidly identifying mHAg loci using a large genotyping data set and LCLs from the International HapMap Project for genome-wide association analysis.[13-15]

Methods

Cell lines and CTL clones

The HapMap LCL samples were purchased from the Coriell Institute (Camden, NJ). All LCLs were maintained in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Because the recognition of a mHag requires presentation on a particular type of HLA molecule, the LCLs were stably transduced with a retroviral vector encoding the restriction HLA cDNA for a given CTL clone when necessary.[16]

CTL lines were generated from recipient peripheral blood mononuclear cells obtained after transplantation by stimulation with those harvested before HSCT after irradiation (33 Gy), and thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. Recombinant human inerleukin-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,17] HLA restriction was determined by conventional CRAs against a panel of LCLs sharing HLA alleles with the CTLs. All clinical samples were collected based on a protocol approved by the Institutional Review Board Committee at Aichi Cancer Center and the University of Tokyoand after written informed consent was obtained in accordance with the Declaration of Helsinki.

Immunophenotyping of HapMap LCLs and high-density genome-wide scanning of mHag loci

Case ($mHag^+$) - control ($mHag^-$) LCL panels were generated by screening corresponding restriction HLA-transduced CHB and JPT HapMap LCL panels with each CTL clone using CRAs. Briefly, target cells were labeled with 0.1 mCi of ^{51}Cr for 2 hours, and 10^3 target cells per well were mixed with CTL at a predetermined E/T ratio in a standard 4-hour CRA. All assays were performed at least in duplicate. The percent specific lysis was calculated by ((Experimental cpm - Spontaneous cpm) / (Maximum cpm - Spontaneous cpm)) × 100. After normalization by dividing their percent specific lysis values by that of positive control LCL (typically recipient-derived LCL corresponding to individual CTL clones), the mHag status of each HapMap LCL was defined as positive, negative, or undetermined.

To identify mHag loci, we performed association tests for all the Phase II HapMap SNPs, by calculating χ^2 test statistics based on 2 × 2 contingency tables with regard to the mHag status as measured by CRA and the HapMap genotypes (presence or absence of a particular allele) at each locus. χ^2 were calculated for the 2 possible mHag alleles at each locus and the larger value was adopted for each SNP. While different test statistics may be used showing different performance, the χ^2 statistic is most convenient for the purpose of power estimation as described below. The maximum value of the χ^2 statistics was evaluated against the thresholds empirically calculated from 100 000 random permutations within a given LCL set. The program was written in C++ and will run on a unix clone. It will be freely distributed on request. Computation of the statistics was performed within several seconds on a Macintosh equipped with 2 × quadcore 3.2 GHz Zeon processors (Apple, Cupertino, CA), although 100 000 permutations took several hours on average.

Evaluation of the power of association tests using HapMap samples

The genotyping data of the Phase II HapMap[14] were obtained from the International HapMap Project website (http://www.hapmap.org/genotypes/latest_ncbi_build35), among which we used the nonredundant data sets (excluding SNPs on the Y chromosome) from 60 CEU (Utah residents with ancestry from northern and western Europe) parents, 60 YRI (Yoruba in Ibadan, Nigeria) parents, and the combined set of 45 JPT (Japanese in Tokyo, Japan) and 45 CHB (Han Chinese in Beijing, China) unrelated people. They contained 3 901 416 (2 624 947 polymorphic), 3 843 537 (295 293 polymorphic), and 3 933 720 (2 516 310 polymorphic) SNPs for CEU, YRI, and JPT + CHB, respectively.

To evaluate the power, we first assumed that the Phase II HapMap SNP set contains the target SNP of the relevant mHag or its complete proxies, and that the immunologic assays can completely discriminate i $mHag^+$ and j $mHag^-$ HapMap LCLs. Under this ideal condition, the test statistic, or χ^2, for these SNPs takes a definite value, $f(i,j) = i+j$, which was compared with the maximum χ^2 value, or its distribution, under the null hypothesis, that is, no SNPs within the Phase II HapMap set should be associated with the mHag locus. Unfortunately, the latter distribution cannot be calculated in an explicit analytical form but needs to be empirically determined based on HapMap data, because Phase II HapMap SNPs are mutually interdependent due to extensive linkage disequilibrium within human populations. For this purpose, we simulated 10 000 case-control panels by randomly choosing i $mHag^+$ and j $mHag^-$ HapMap LCLs for various combinations of (i,j) and calculated the maximum χ^2 values (χ^2_{max}) for each panel to identify those (i,j) combinations, in which $f(i,j)$ exceeds the upper 1 percentile point of the simulated 10 000 maximum values, $g(i,j)^{P=.01}$.

When proxies are not complete (ie, $r^2 < 1$), the expected values will be decayed by the factor of r^2, and further reduced due to the probabilities of false positive (f_P) and negative (f_N) assays, and expressed as $\hat{f}(i,j) = (i + j) \times \hat{r}^2$ through an apparent r^2 ($\hat{r}^2$) as provided in formula 1.[1] Under given probabilities of assay errors and maximum LD strength between markers and the mHag allele, we can expect to identify target mHag loci for those (i,j) sets that satisfy $\hat{f}(i,j) > g(i,j)^{P=.01}$.

Empirical estimation of distributions of r^2

The maximum r^2 value (r^2_{max}) between a given mHag allele and one or more Phase II HapMap SNPs was estimated based on the observed HapMap data set. Each Phase II HapMap SNP was assumed to represent a target mHag allele, and the (r^2_{max}) was calculated, taking into account all the Phase II HapMap SNPs less than 500 kb apart from the target SNP.

Confirmatory genotyping

Genotyping was carried out either by TaqMan MGB technology (Applied Biosystems, Foster City, CA) with primers and probes for HA-1 mHag according to the manufacturer's protocol using an ABI 7900HT with the aid of SDS version 2.2 software (Applied Biosystems) or by direct sequencing of amplified cDNA for the *SLC1A5* gene. cDNA was reverse transcribed from total RNA extracted from LCLs, and polymerase chain reaction (PCR) was conducted with cDNA with the corresponding primers. Amplified DNA samples were sequenced using BigDye Terminator version 3.1 (Applied Biosystems). The presence or absence (deletion) of the *UGT2B17* gene was confirmed by genomic PCR with 2 primer sets for exons 1 and 6 as described previously[18] using DNA isolated from LCLs of interest.

Epitope mapping

A series of deletion mutant cDNAs were designed and cloned into pcDNA3.1/V5-His TOPO plasmid (Invitrogen, Carlsbad, CA). Thereafter, 293T cells that had been transduced with restricting HLA class I cDNA for individual CTL clones were transfected with each of the deletion mutants and cocultured with the CTL clone overnight to induce interferon (IFN)-γ release, which was then evaluated by enzyme-linked immunosorbent assay (ELISA) as previously described.[9]

For *SLC1A5*, expression plasmids encoding full-length cDNA and the exon 1 of recipient and donor origin were first constructed because only the SNP in the exon 1 was found to be concordant with susceptibility to CTL-3B6. Next, amino (N)- and (carboxyl) C-terminus–truncated mini-genes encoding polypeptides around the polymorphic amino acid defined by the SNP were amplified by PCR from *SLC1A5* exon 1 cDNA as template and cloned into the above plasmid. The constructs all encoded a Kozak

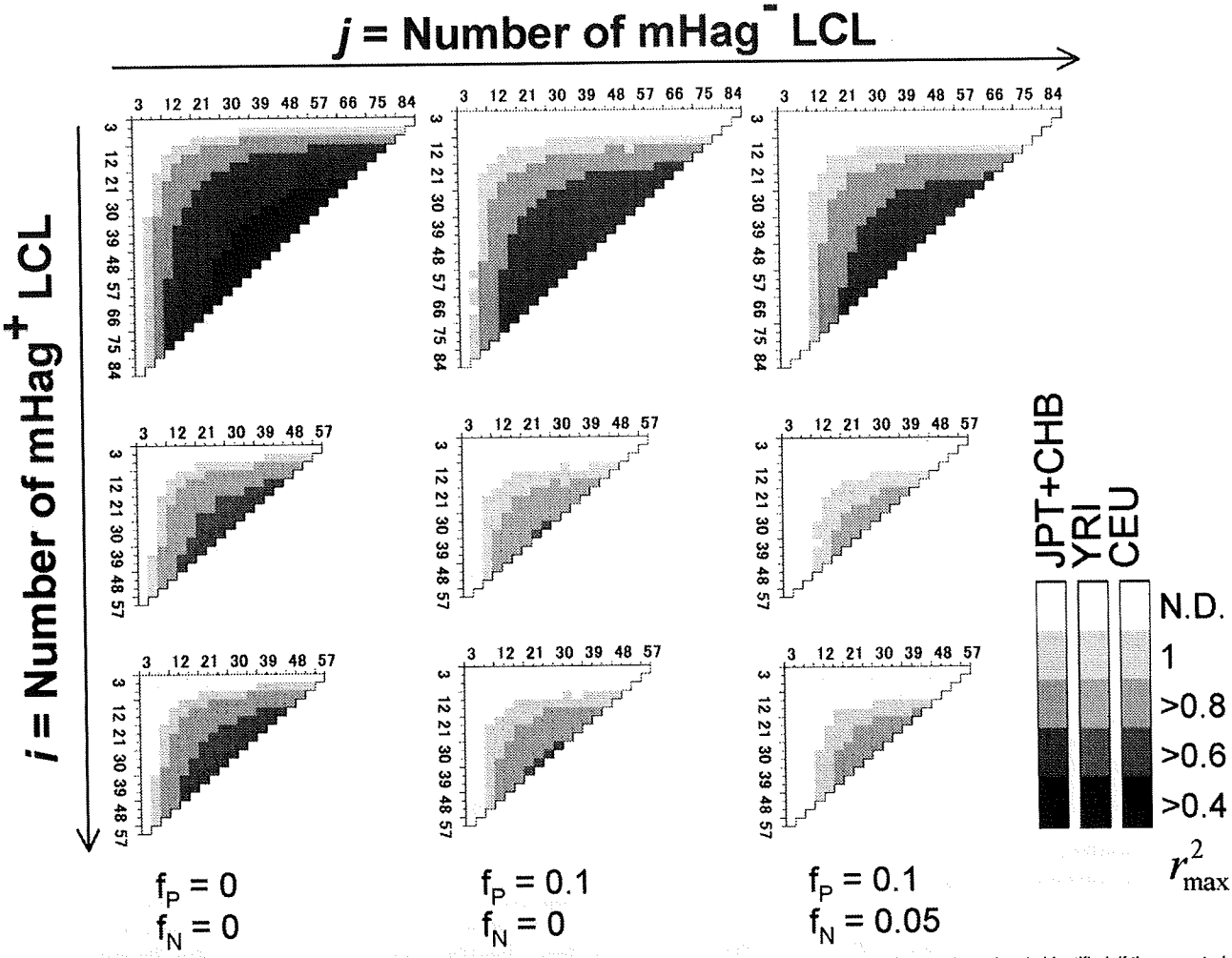

Figure 1. Numbers of positive and negative LCLs required for successful mHag mapping. The target locus was assumed to be uniquely identified, if the expected χ^2 value for the target SNP ($\tilde{\chi}(i,j)$, see Document S1) exceeded the upper 1 percentile point of the maximum χ^2 values in 10 000 simulated case-control panels ($g(i,j)^{P=.01}$). Combinations of the numbers of mHag$^+$ (vertical coordinates) and mHag$^-$ (horizontal coordinates) samples satisfying the above condition are shown in color gradients corresponding to different max r^2 values between the target SNP and one or more nearby Phase II HapMap SNPs (r^2_{max}), ranging from 0.4 to 1.0. Calculations were made for 3 HapMap population panels, CHB + JPT (top), YRI (middle), and CEU (bottom) and for different false positive and negative rates, $f_P = f_N = 0$ (left), $f_P = 0.1, f_N \cong 0$ (middle), and $f_P = 0.1, f_N = 0.05$ (right), considering the very low false negative assays for CRAs.

sequence and initiator methionine (CCACC-ATG) and for C-terminus deletions a stop codon (TAG).

For *UGT2B17*, a series of C-terminus deletion mutants with approximately 200 bp spacing was first constructed as above. For further mapping, N-terminus deletion mutants were added to the region that was deduced to be potentially encoding the CTL-1B2 epitope. For prediction of a CTL epitope, the HLA Peptide Binding Predictions algorithm on the BioInformatics & Molecular Analysis Section (BIMAS) website (http://www.bimas.cit.nih.gov/molbio/hla_bind/)[19] was used because HLA-A*0206 has a similar binding motif to that of A*0201.

Epitope reconstitution assay

The candidate mHag epitopes and allelic counterpart peptides (in case of SLC1A5) were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled mHag$^-$ donor LCL were incubated with graded concentrations of the peptides and then used as targets in standard CRAs.

Results and discussion

Statistical approach and estimation of potential overfitting

We reasoned that the mHag locus recognized by a given CTL clone could be defined by grouping LCLs from a HapMap panel into mHag$^+$ and mHag$^-$ subpanels according to their susceptibility to lysis by the CTL clone and then performing an association scan using the highly qualified HapMap data set containing more than 3 000 000 SNP markers. The relevant genetic trait here is expected to show near-complete penetrance, and the major concern with this approach arises from the risk of overfitting observed phenotypes to one or more incidental SNPs with this large number of HapMap SNPs under the relatively limited size of freedom due to small numbers of independent HapMap samples (90 for JPT + CHB and 60 for CEU and YRI, when not including their offspring).[13]

To address this problem, we first estimated the maximum sizes of the test statistics (here, χ^2 values) under the null hypothesis (ie, no associated SNPs within the HapMap set) by simulating 10 000 case-control HapMap panels under different experimental conditions, and compared them with the expected size of test statistic values from the marker SNPs associated with the target SNP, assuming different linkage disequilibrium (LD), or r^2 values in between. As shown in Figure 1, the possibility of overfitting became progressively reduced as the number of LCLs increased, which would allow for identification of the target locus in a broad range of r^2 values, except for those mHags having very low minor allele frequencies (MAF) below

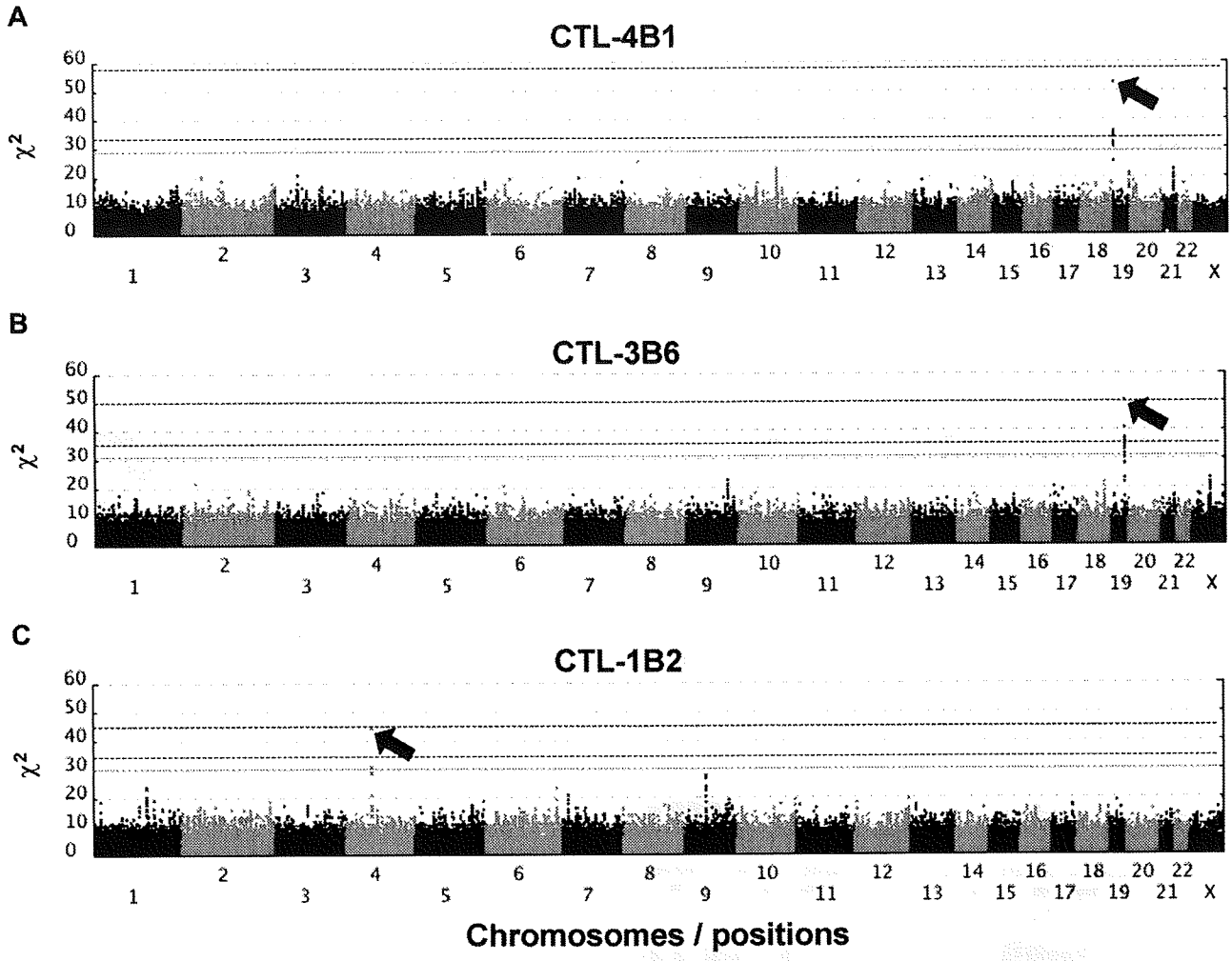

Figure 2. Genome-wide scanning to identify chromosome location of mHag. χ^2 values were plotted against positions on each chromosome for each of 3 mHags recognized by CTL-4B1 (A), CTL-3B6 (B), and CTL-1B2 (C). Chromosomes are displayed in alternating colors. Threshold χ^2 values corresponding to the genome-wide $P = 10^{-3}$ (dark blue) and 10^{-2} (light blue), as empirically determined from 100 000 random permutations, are indicated by broken lines, while the theoretically possible maximum values are shown with red broken lines. The highest χ^2 value in each experiment is indicated by a red arrow.

approximately 0.05. According to our estimation using the Phase II HapMap data (see "Methods"), the majority (> 90%) of common target SNPs (MAF > ~ 0.05) could be captured by one or more HapMap SNPs with more than 0.8 of r^2 (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), ensuring a high probability of detecting an association (Figure 1 left panels). The simulation of pseudo-Phase II sets generated from the ENCODE regions provided a similar estimation.[13] False positive and negative immunophenotyping results could also complicate the detection, reducing the expected test statistics through the "apparent" r^2 values ($\hat{r}^2$), as defined by

$$\hat{r}^2 = r^2 \times \frac{(1 - f_P - f_N)^2}{(1 - f_P + f_N q)(1 - f_N + f_P/q)} \quad (1)$$

where f_P, f_N, and q represent false typing probabilities with positive and negative LCL panels, and the ratio of the positive to the negative LCL number, respectively. However, the high precision of cytotoxicity assays (f_P ~ < 0.1, f_N ~ = 0) limits this drawback from the second term to within acceptable levels and allows for sensitive mHag locus mapping with practical sample sizes (Figure 1 middle and right panels), suggesting the robustness of our novel approach.

Evaluation of the detection power for known mHags

Based on these considerations, we then assessed whether this approach could be used to correctly pinpoint known mHag loci (Table S1). Because the relevant mHag alleles are common SNPs and directly genotyped in the Phase II HapMap set, or if not, located within a well-defined LD block recognized in this set (Figure S2), their loci would be expected to be uniquely determined with an acceptable number of samples, as predicted from Figure 1. To test this experimentally, we first mapped the locus for HA-1^H mHag[7] by evaluating recognition of the HLA-A*0206–transduced HapMap cell panel with HLA-A*0206–restricted CTL-4B1.[20] After screening 58 well-growing LCLs from the JPT + CHB panel with CRAs using CTL-4B1 (Figure S3A; Tables S2,S3), we obtained 37 mHag$^+$ and 21 mHag$^-$ LCLs, which were tested for association at 3 933 720 SNP loci. The SNP (rs1801284) encoding the mHag is located within a HapMap LD block on chromosome 19q13.3, but is not directly genotyped within this data set. The genome-wide scan clearly indicated a unique association with the HA-1^H locus within the *HMHA1* gene, showing a peak χ^2 statistic of 52.8 (not reached in 100 000 permutations) at rs10421359 (Figures 2A,3A; Tables S2,S3).

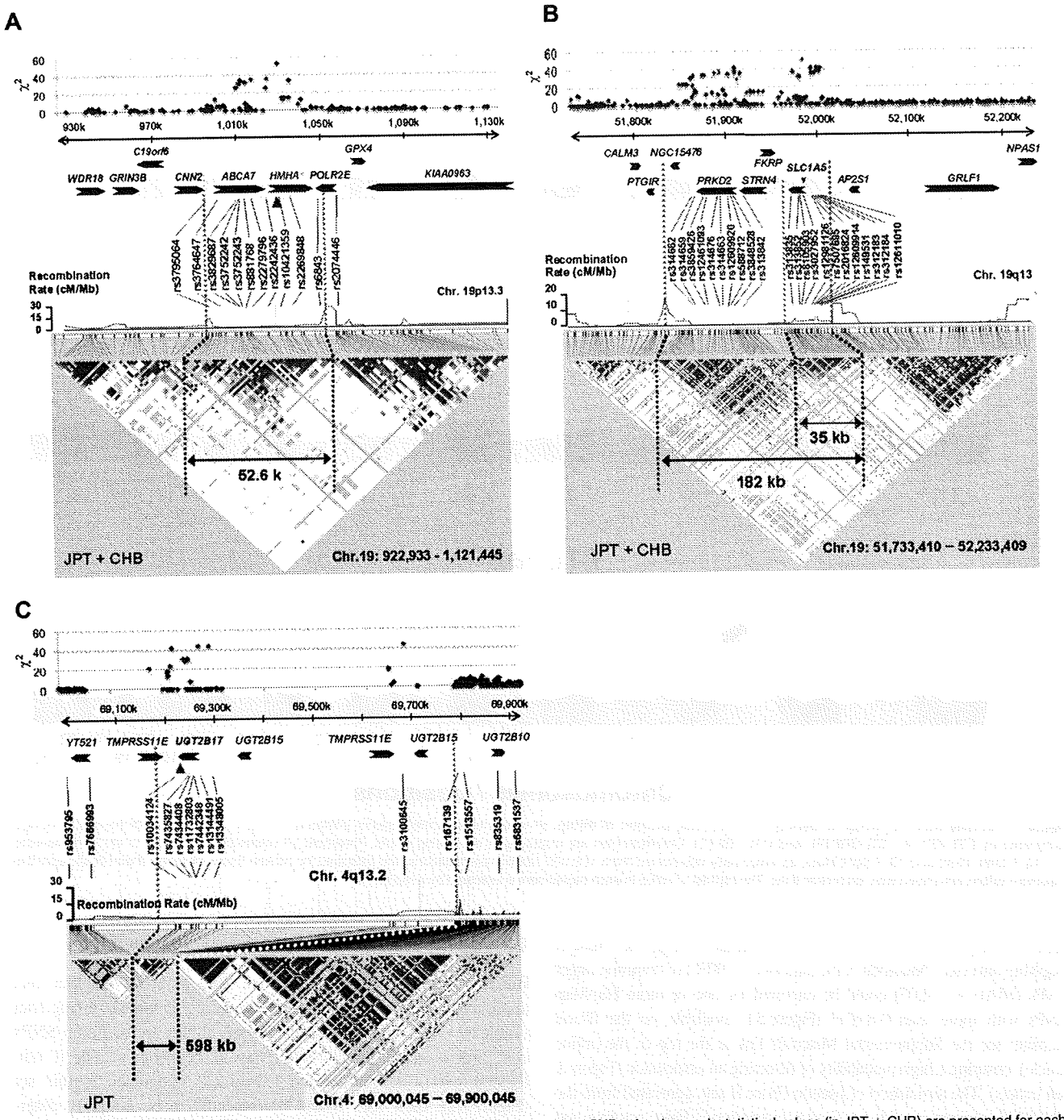

Figure 3. Regions of mHag loci identified by HapMap scanning. LD structures around the SNPs showing peak statistical values (in JPT + CHB) are presented for each mHag locus identified with (A) CTL-4B1, (B) CTL-3B6, and (C) CTL-1B2. Regional χ^2 plots are also provided on the top of each panel. LD plots in pairwise D's with recombination rates along the segment were drawn with HaploView software version 4.0 (http://www.broad.mit.edu/mpg/haploview/). The size and location of each LD block containing a mHag locus are indicated within the panels. Significant SNPs (blue letters), as well as other representative SNPs, are shown in relation to known genes. The positions of the SNPs showing the highest statistic values (red letters) are indicated by red arrowheads.

Identification of novel mHags

We next applied this method to mapping novel mHags recognized by CTL clone 3B6, which is HLA-B*4002–restricted; and CTL clone 1B2, which is HLA-A*0206–restricted. Both clones had been isolated from peripheral blood samples of post-HSCT different patients. In preliminary CRAs with the JPT + CHB panel, allele frequencies of target mHags for CTL-3B6 and CTL-1B2 in this panel were estimated as approximately 25% and approximately 45%, respectively (data not shown). After screening 72 JPT + CHB LCLs with CTL-3B6, 36 mHag$^+$ and 14 mHag$^-$ LCLs were obtained, leaving 22 LCLs undetermined based on empirically determined thresholds (> 51% for mHag + LCLs and < 11% for mHag-LCLs; Figure S3B, Tables S2,S4). As shown in Figure 2B, the χ^2 statistics calculated from the immunophenotyping data produced discrete peaks in the LCL sets. The peak in chromosome 19q13.3 for the CTL-3B6 set showed the theoretically maximum χ^2 value of 50 (not reached in 100 000 permutations) at rs3027952, which was mapped within a small LD block of

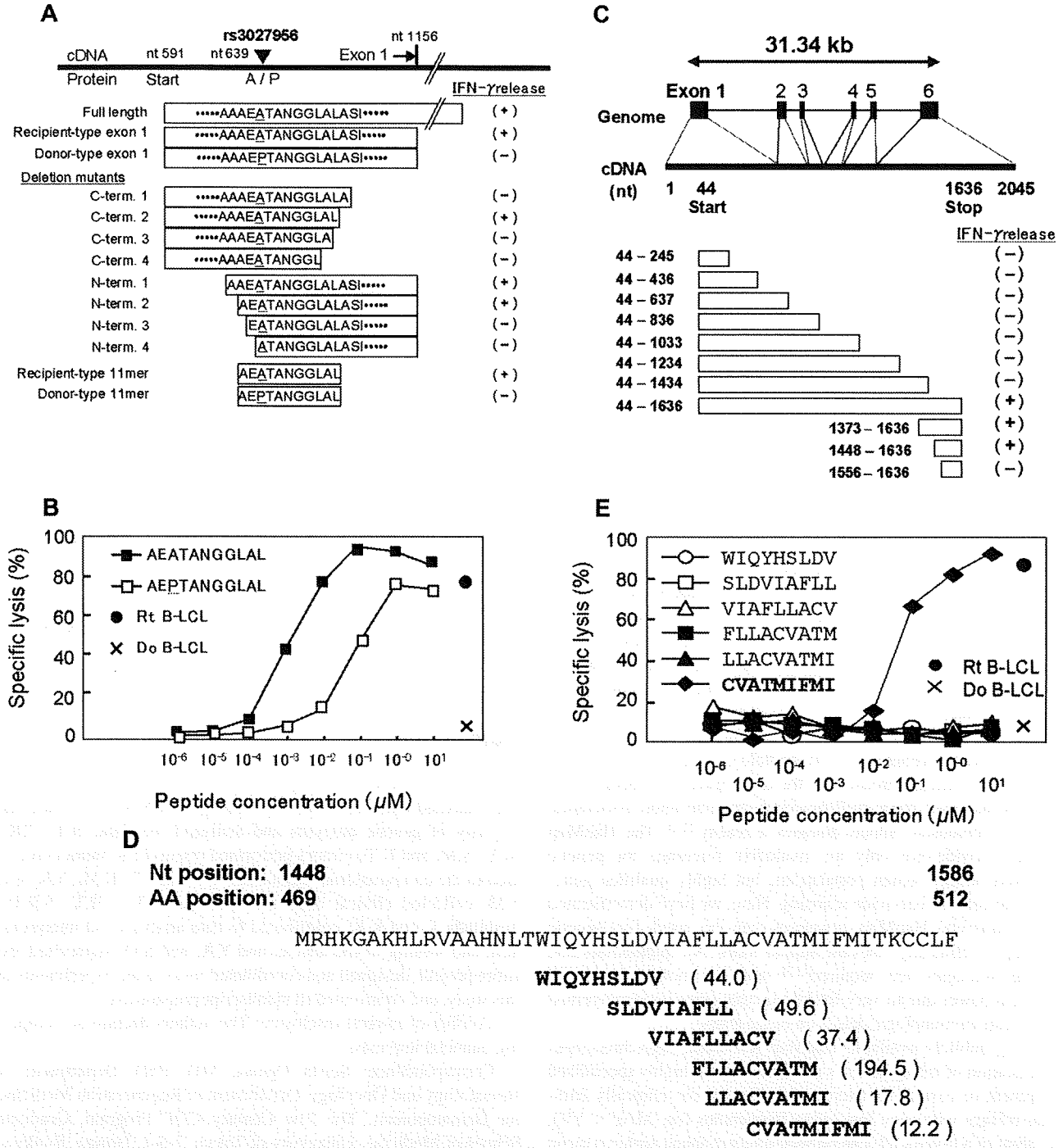

Figure 4. Epitope mapping. (A) Determination of the *SLC1A5* epitope by deletion mapping. Plasmids encoding recipient full-length *SLC1A5*, exon 1 of recipient and donor, exon 1 with various N- and C-terminus deletions around the amino acid encoded by SNP rs51983014, and minigenes encoding AEATANGGLAL and its allelic counterpart AEPTANGGLAL were constructed and transfected into HLA-B*4002–transduced 293T cells. Interferon (IFN)-γ was assessed by ELISA (right column) after coculture of CTL-3B1 with 293T transfectants. (B) Epitope reconstitution assay with synthetic undecameric peptides, AEATANGGLAL and AEPTANGGLAL. (C) Structure of the *UGT2B17* gene and screening of *UGT2B17* cDNA and deletion mutants. HLA-A*0206–transduced 293T cells were transfected with each plasmid and cocultured with CTL-2B1. IFN-γ production from CTL-1B2 (right column) indicated that the epitope was likely encoded by nucleotides 1448-1586, including 30 nucleotides from position 1566 that could potentially encode part of the epitope. (D) Epitope prediction using the HLA Peptide Binding Predictions algorithm.[19] Because HLA-A*0201 and -A*0206 have similar peptide binding motifs,[30] the algorithm for HLA-A*0201 was used to predict candidate epitopes recognized by CTL-1B2. Values in parentheses indicate the predicted half-time of dissociation. (E) Epitope reconstitution assays with graded concentrations of synthetic nonameric peptides shown in panel D.

approximately 182 kb, or more narrowly within its 35 kb sub-block containing a single gene, *SLC1A5*, as a candidate mHag gene (Figure 3B). In fact, when expressed in 293T cells with HLA-B*4002 transgene, recipient-derived, but not donor-derived, *SLC1A5* cDNA was able to stimulate IFN-γ secretion from CTL-3B6 (Figure 4A), indicating that *SLC1A5* actually encodes the target mHag recognized by CTL-3B6. Conventional epitope mapping using a series of deletion mutants of *SLC1A5* cDNA finally identified an undecameric peptide, AEATANGGLAL, as the minimal epitope (Figure 4A). The donor-type AEPTANGGLAL induced IFN-γ with a 2-log lower efficiency, suggesting that AEPTANGGLAL may not be transported efficiently into the ER

because endogenous expression of a minigene encoding AEPTANGGLAL was not recognized by CTL-3B1 (Figure 4B). Unfortunately, although the peak statistic value showed the theoretically maximum value for this data set, it did not conform to the relevant SNP for this mHag (rs3027956) due to high genotyping errors of the HapMap data at this particular SNP. However, the result of our resequencing showed complete concordance with the presence of the rs3027956 SNP and recognition in the cytotoxicity assay (Table S4).

Similarly, 13 $mHag^+$ and 32 $mHag^-$ LCLs were identified from the screening of 45 JPT LCLs from the same panel using CTL-1B2 (Figure S3C; Tables S2,S5). The χ^2 statistics calculated from the immunophenotyping data produced bimodal discrete peaks with this LCL set. The target locus for the mHag recognized by CTL-1B2 was identified at a peak (max $\chi^2 = 44$, not reached in 100 000 permutations) within a 598-kb block on chromosome 4q13.1, coinciding with the locus for a previously reported mHag, *UGT2B17*[18] (Figures 2C, 3C). In fact, our epitope mapping using *UGT2B17* cDNA deletion mutants (Figure 4C), prediction of candidate epitopes by HLA-binding algorithms[19] (Figure 4D) and epitope reconstitution assays (Figure 4E), successfully identified a novel nonameric peptide, CVATMIFMI. Of particular note, this mHag was not defined by a SNP but by a CNV (ie, a null allele[18]) that is in complete LD with the SNPs showing the maximum χ^2 value (Table S5). Transplanted T cells from donors lacking both *UGT2B17* alleles are sensitized in recipients possessing at least 1 copy of this gene.[18] Although LD between SNPs and CNVs has been reported to be less prominent,[21] this is an example where a CNV trait could be captured by a SNP-based genome-wide association study.

The recent generation of the HapMap has had a profound impact on human genetics.[13,15] In the field of medical genetics, the HapMap is a central resource for the development of theories and methods that have made well-powered, genome-wide association studies of common human diseases a reality.[22-28] The HapMap samples provide not only an invaluable reference for genetic variations within human populations, but highly qualified genotypes that enable gene-wide scanning. Here, we have demonstrated how effectively HapMap resources can be used for genetic mapping of clinically relevant human traits. No imputations and tagging strategies are required[25,28] and the potential loss of statistical power due to very limited sample sizes is circumvented by accurate immunologic detection of the traits.

Using publicly available HapMap resources, high-throughput identification of mHag genes is possible without highly specialized equipment or expensive microarrays. Except for clinically irrelevant mHags with very low allele frequencies (eg, MAF < 5%), the target of a given CTL can be sensitively mapped within a mean LD block size, typically containing just a few candidate genes. The methodology described here will facilitate construction of a large panel of human mHags including those presented by MHC class II molecules, and promote our understanding of human allo-immunity and development of targeted allo-immune therapies for hematologic malignancies.[1,2] The HapMap scan approach may be useful for exploring other genetic traits or molecular targets (eg, differential responses to some stress or drugs), if they can be discriminated accurately through appropriate biologic assays. In this context, the recent report that we may reprogram the fate of terminally differentiated human cells[29] is encouraging, indicating possible exploration of genotypes that are relevant to cell types other than immortalized B cells.

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Authorship

Contribution: M.K. performed most of immunologic experiments and analyzed data and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T., T.K., M.Y., S.M. and K.Tsujimura performed research; K.Taura contributed to the computational simulation; Y.I., Taro T., K.M., Y.K. and Y.M. collected clinical data and specimens; T.I., H.T., S.R.R., Toshitada T. and K.K. contributed to data analysis and interpretation, and writing of the article; and Y.A. and S.O. supervised the entire project, designed and coordinated most of the experiments in this study, and contributed to manuscript preparation.

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References

1. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4:371-380.
2. Spierings E, Goulmy E. Expanding the immunotherapeutic potential of minor histocompatibility antigens. J Clin Invest. 2005;115:3397-3400.
3. de Rijke B, van Horssen-Zoetbrood A, Beekman JM, et al. A frameshift polymorphism in P2X5 elicits an allogeneic cytotoxic T lymphocyte response associated with remission of chronic myeloid leukemia. J Clin Invest. 2005;115:3506-3516.
4. Marijt WA, Heemskerk MH, Kloosterboer FM, et al. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. Proc Natl Acad Sci U S A. 2003;100:2742-2747.
5. Spierings E, Hendriks M, Absi L, et al. Phenotype frequencies of autosomal minor histocompatibility antigens display significant differences among populations. PLoS Genet. 2007;3:e103.
6. Brickner AG, Warren EH, Caldwell JA, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. J Exp Med. 2001;193:195-206.
7. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science. 1998;279:1054-1057.

8. Dolstra H, Fredrix H, Maas F, et al. A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. J Exp Med. 1999; 189:301-308.
9. Kawase T, Akatsuka Y, Torikai H, et al. Alternative splicing due to an intronic SNP in HMSD generates a novel minor histocompatibility antigen. Blood. 2007;110:1055-1063.
10. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. J Exp Med. 2003;197: 1489-1500.
11. Kawase T, Nanya Y, Torikai H, et al. Identification of human minor histocompatibility antigens based on genetic association with highly parallel genotyping of pooled DNA. Blood. 2008;111:3286-3294.
12. Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science. 1996;273:1516-1517.
13. The International HapMap Consortium. The International HapMap Project. Nature. 2003;426:789-796.
14. The International HapMap Consortium. A second generation human haplotype map of over 3.1 million SNPs. Nature. 2007;449:851-861.
15. The International HapMap Consortium. A haplotype map of the human genome. Nature. 2005; 437:1299-1320.
16. Akatsuka Y, Goldberg TA, Kondo E, et al. Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines. Tissue Antigens. 2002;59:502-511.
17. Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J Immunol Methods. 1990;128:189-201.
18. Murata M, Warren EH, Riddell SR. A human minor histocompatibility antigen resulting from differential expression due to a gene deletion. J Exp Med. 2003;197:1279-1289.
19. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol. 1994;152:163-175.
20. Torikai H, Akatsuka Y, Miyauchi H, et al. The HLA-A*0201-restricted minor histocompatibility antigen HA-1H peptide can also be presented by another HLA-A2 subtype, A*0206. Bone Marrow Transplant. 2007;40:165-174.
21. Redon R, Ishikawa S, Fitch KR, et al. Global variation in copy number in the human genome. Nature. 2006;444:444-454.
22. Barrett JC, Cardon LR. Evaluating coverage of genome-wide association studies. Nat Genet. 2006;38:659-662.
23. Nannya Y, Taura K, Kurokawa M, Chiba S, Ogawa S. Evaluation of genome-wide power of genetic association studies based on empirical data from the HapMap project. Hum Mol Genet. 2007;16:2494-2505.
24. Pe'er I, de Bakker PI, Maller J, Yelensky R, Altshuler D, Daly MJ. Evaluating and improving power in whole-genome association studies using fixed marker sets. Nat Genet. 2006;38:663-667.
25. Marchini J, Howie B, Myers S, McVean G, Donnelly P. A new multipoint method for genome-wide association studies by imputation of genotypes. Nat Genet. 2007;39:906-913.
26. Altshuler D, Daly M. Guilt beyond a reasonable doubt. Nat Genet. 2007;39:813-815.
27. Bowcock AM. Genomics: guilt by association. Nature. 2007;447:645-646.
28. de Bakker PI, Burtt NP, Graham RR, et al. Transferability of tag SNPs in genetic association studies in multiple populations. Nat Genet. 2006;38: 1298-1303.
29. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131: 861-872.
30. Sudo T, Kamikawaji N, Kimura A, et al. Differences in MHC class I self peptide repertoires among HLA-A2 subtypes. J Immunol. 1995;155: 4749-4756.

Hematopoietic stem cell transplantation for core binding factor acute myeloid leukemia: t(8;21) and inv(16) represent different clinical outcomes

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We analyzed 338 adult patients with acute myeloid leukemia (AML) with t(8;21) and inv(16) undergoing stem cell transplantation (SCT) who were registered in the Japan Society for Hematopoietic Cell Transplantation database. At 3 years, overall survival (OS) of patients with t(8;21) and inv(16) was 50% and 72%, respectively ($P = .002$). Although no difference was observed when restricted to allogeneic SCT in first complete remission (CR; 84% and 74%), OS of patients with t(8;21) and inv(16) undergoing allogeneic SCT in second or third CR (45% and 86% at 3 years; $P = .008$) was different. OS was not different between patients in first CR who received allogeneic SCT and those who received autologous SCT for both t(8;21) AML (84% vs 77%; $P = .49$) and inv(16) AML (74% vs 59%; $P = .86$). Patients with inv(16) not in CR did better after allogeneic SCT than those with t(8;21) (70% and 18%; $P = .03$). Patients with t(8;21) and inv(16) should be managed differently as to the application of SCT. SCT in first CR is not necessarily recommended for inv(16). For t(8;21) patients in first CR, a prospective trial is needed to clarify the significance of autologous SCT and allogeneic SCT over chemotherapy. (Blood. 2009;113:2096-2103)

Introduction

Core binding factor (CBF) acute myeloid leukemia (AML) including t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22) [t(8;21) and inv(16)] is considered to be a favorable cytogenetic subgroup in clinical studies.[1-4] Patients with t(8;21) and inv(16) have shown a markedly improved outcome with repetitive use of high-dose cytarabine.[5-13] However, the major treatment failure is disease recurrence.[14-16] These patients frequently become stem cell transplantation (SCT) candidates.

Both t(8;21) and inv(16) AMLs are associated with disruption of genes encoding subunits of the CBF, a heterodimeric transcriptional factor involved in the regulation of hematopoiesis.[17,18] Although these 2 different cytogenetics also share common clinical characteristics, they are associated with different clinical features such as morphologic presentation and immunophenotypic marker expression.[19]

Several reports demonstrated inferior outcome of t(8;21) compared with inv(16), but the number of patients who underwent transplantation was limited.[14,15,20] A recent study from the Dana-Farber Cancer Institute reported that both patients with t(8;21) and inv(16) de novo AML who underwent allogeneic transplantation performed favorably compared with other karyotypes.[21] To identify the survival data and prognostic factors among the CBF leukemia population who received SCT, we conducted a retrospective analysis using a Japanese multi-institution database with a large number of patients.

Methods

Study population

A total of 2802 adult patients who underwent autologous or allogeneic SCT from 1996 and 2004 for AML were registered in the Japan Society for Hematopoietic Cell Transplantation (JSHCT) database. Patients who underwent SCT from unrelated donors were registered in the different registry in the study period, but not all of the patients undergoing unrelated SCT were registered in the JSHCT database. Demographic, diagnostic, clinical, cytogenetics, induction, and outcome information were collected for each patient, and were sent to a central registration center. Cytogenetic studies were performed in each center, but a central review of cytogenetic analysis was not performed.

Patients with de novo AML aged 16 to 70 years who received hematopoietic SCT as the first transplant were included in the study. No patients with prior history of autologous or allogeneic SCT were included in the study. Of the remaining 2164 patients, 178 patients with t(15;17) or PML/RARα were excluded from the analysis below (Table 1). Finally, of the 1986 patients included in the analysis, 255 were reported to have t(8;21) abnormality, and 83 to have inv(16). A total of 194 patients had no available cytogenetic data. The remaining 1454 patients with normal karyotype and other cytogenetic abnormalities were further coded and analyzed according to published Southwest Oncology Group (SWOG) criteria.[3] The intermediate risk category included patients characterized by +8, −Y, +6, del(12p), or normal karyotype. The unfavorable risk category was defined by the presence of one or more of −5/del(5q), −7/del(7q), abn 3q, 11q, 20q, or

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Table 1. Cytogenetic risk groups of patients with AML who received autologous SCT and allogeneic SCT

Cytogenetic risk groups	No. patients		
	Auto-SCT	Allo-SCT	Total
t(8;21)	61	194	255
inv(16)	17	66	83
t(15;17)*	65	113	178
Intermediate	140	749	889
Unfavorable	35	325	360
Unknown			
Unknown cytogenetic risk	27	178	205
No available cytogenetic data	44	150	194
Total	389	1775	2164

Auto-SCT indicates autologous stem cell transplantation; Allo-SCT, allogeneic stem cell transplantation.

*Patients with t(15;17) were excluded from the analysis.

21q, del(9q), t(6;9), t(9;22), abn 17p, and complex karyotypes defined as 3 or more abnormalities. Patients with other cytogenetic aberrations were considered an unknown risk group, and were analyzed together with 194 patients with no cytogenetic data.

This study was approved by the Committee for Nationwide Survey Data Management of the JSHCT. Informed consent was obtained in accordance with the Declaration of Helsinki.

Transplantation

A total of 1662 patients underwent allogeneic SCT, and 324 underwent autologous SCT. Patients were treated with various conditioning regimens, but most of those who underwent autologous transplantation received non–total body irradiation (TBI) regimens (97%), including busulfan (BU), cytarabine (CA), and etoposide. The most frequently used conditioning regimens before allogeneic SCT were cyclophosphamide (Cy) plus TBI (n = 327 patients), and BU plus Cy (n = 267). Conditioning regimens before allogeneic SCT also included more intensified regimens such as CA plus Cy plus TBI (n = 262) and BU plus Cy plus TBI (n = 146), or reduced-intensity conditioning regimens with fludarabine (n = 241) or cladribine (n = 19).

Stem cell sources for allogeneic SCT were bone marrow in 871 patients, peripheral blood stem cell in 570 patients, bone marrow plus peripheral blood stem cell in 23 patients, and cord blood in 190 patients. A total of 1242 patients underwent allogeneic SCT from a related donor, and 404 patients underwent SCT from an unrelated donor.

Of the 1637 patients who had available data, 74% received transplants from human leukocyte antigen (HLA)–matched donors. Among patients who received unrelated bone marrow transplants, 156 patients were HLA genotypically matched and 51 were HLA mismatched. HLA data for 39 mismatched unrelated bone marrow transplantation patients were available. A total of 32 patients were one locus mismatched, and 7 patients were 2 loci mismatched. Among patients receiving unrelated cord blood transplants, 19 patients were serologically HLA matched and 170 patients were mismatched. HLA incompatibility was 5 of 6 HLA matched in 57 patients, 4 of 6 HLA matched in 99 patients, 3 of 6 HLA matched in 7 patients, and 1 of 6 HLA matched in 1 patient.

Graft-versus-host disease (GVHD) prophylaxis mostly consisted of methotrexate and a calcineurin inhibitor, either cyclosporin A or tacrolimus. Several other prophylaxes include mycophenolate mofetil, antithymocyte globulin, and $CD34^+$ selection. The incidence of acute GVHD was evaluated in 1488 patients who survived more than 28 days, and chronic GVHD was evaluated in 1302 patients who survived more than 100 days after allogeneic SCT. GVHD was evaluated in each center.

Statistical analysis

Correlation between the 2 groups was examined with the chi-square test, Fisher exact test, and the Mann-Whitney *U* test. Disease-free survival (DFS) was calculated from the date of transplantation until the date of relapse or the date of death in CR. Patient survival data were analyzed with the method of Kaplan and Meier and compared by the log-rank test.

Univariate and multivariate analyses for OS were performed with the aid of the Cox proportional hazard regression model, and variables were selected with the stepwise method. The following variables were evaluated: age, sex, and disease status at transplantation; CR versus not in CR; the number of induction courses to achieve CR; one course versus more than one course and failure; type of transplantation (allogeneic SCT vs autologous SCT); conditioning regimen (reduced intensity vs myeloablative); TBI regimen or not; and the existence of additional karyotype abnormalities or not. For those who received allogeneic SCT, in addition to these variables, the following were also evaluated: type of GVHD prophylaxis; short-course methotrexate plus cyclosporin A or short methotrexate plus FK506; acute GVHD, grade II to IV or grade III to IV; chronic GVHD; HLA mismatch; donor; and donor source. The doses of methotrexate were not surveyed. Each factor was considered to be prognostic if the *P* value was less than .05. Data were analyzed with the Stata 9.2 statistical software (College Station, TX).

Results

Initial characteristics of patients

The median age of all patients with AML in total was 41 years old (range, 16-70 years old). Median follow-up period of living patients was 37.3 months (range, 0.4-108 months). Patients were categorized into 5 cytogenetic subgroups: with t(8;21), with inv(16), intermediate risk cytogenetics, unfavorable cytogenetics, and an unknown risk group. Table 1 shows the number of patients in each cytogenetic subgroup and patients with t(15;17), who were excluded from the analysis.

Characteristics of the patients with CBF who underwent allogeneic SCT or autologous SCT are shown in Table 2. No significant difference was observed between characteristic of 2 groups of patients with CBF who received autologous SCT, except for the initial white blood cell count.

Of the 259 patients with CBF who received allogeneic SCT, significantly more patients with t(8;21) had failed to achieve CR with a single course of induction chemotherapy at diagnosis ($P = .002$), and were not in CR at the time of transplantation ($P < .001$). Among patients in CR at transplantation, the ratio of those in first, second, or third CR was not different between t(8;21) and inv(16) subgroups. Significantly more patients with inv(16) received transplants from an unrelated donor ($P = .004$). Table 3 and Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) summarize the transplantation data of those undergoing allogeneic SCT. More patients with inv(16) received unrelated transplants compared with t(8;21) patients ($P = .004$).

Overall survival

The OS of 1986 patients with AML at 3 years was 48%, and those with t(8;21), inv(16), intermediate, unfavorable, and unknown cytogenetic risks showed OS of 50%, 72%, 52%, 35%, and 45%, respectively ($P < .001$). Figure 1 shows survival curves of patients with AML patients who underwent allogeneic SCT in first CR (Figure 1A), in second or third CR (Figure 1B), or not in CR (Figure 1C), categorized by the cytogenetic abnormalities. Survival data are listed in Table 4. The OS of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown risk undergoing allogeneic SCT in first CR was 84%, 74%, 69%, 53%, and 52%, respectively ($P < .001$), and that of patients undergoing allogeneic-SCT

Table 2. Characteristics of patients with CBF AML

	Auto-SCT			Allo-SCT		
	t(8;21) (n = 61), no.	inv(16) (n = 17), no.	P	t(8;21) (n = 194), no.	inv(16) (n = 66), no.	P
Median age, y (range)	44 (17-68)	37 (19-61)	.59	39 (16-70)	34 (16-64)	.054
Median WBC, g/L (range)	8.8 (0.2-94)	33 (2.1-199)	.02	11 (.6-366)	53 (1.8-284)	< .001
Sex						
Male	41	12	.79	117	40	.93
Female	20	5		74	26	
No. of induction chemotherapy at diagnosis of AML						
1 course	48	15	.72	125	55	.002
> 1 or failure*	11	2		56	7	
Additional cytogenetic abnormalities						
None	53	15	> .999	153	54	.61
Positive	8	2		41	12	
Disease status at SCT						
CR	55	16	> .999	108	52	< .001
Not in CR	6	1		85	11	
CR1	43	13	.98	49	21	.29
CR2	7	1		45	26	
CR3	0	1		5	4	
Conditioning regimen						
TBI	0	1	.22	118	47	.078
Not TBI	61	16		71	16	

Correlation between the two groups was examined.
WBC indicates white blood cell count; g/L, 10^9/L; CR1, first complete remission; and CR2 or 3, second or third CR.
*More than 1 or failure includes patients who did not achieve complete remission after first course of induction chemotherapy, and those who were resistant to induction chemotherapy.

in second or third CR was 45%, 86%, 57%, 44%, and 64%, respectively ($P = .09$). OS of patients undergoing allogeneic SCT not in CR was 18%, 70%, 25%, 15%, and 18%, respectively ($P = .003$).

Table 3. Summary of allogeneic SCT

	t(8;21) (n = 194), no.	inv(16), (n = 66), no.	P
Conditioning regimen			
RIST	31	9	.66
Myeloablative	161	56	
GVHD prophylaxis*			
sMTX+CyA	136	48	.78
sMTX+FK	20	8	
HLA			
Match	146	47	.5
Mismatch	45	18	
Donor			
Related	161	44	.004
Unrelated	32	22	
Stem cell source			
BM	101	40	.27
PB	72	17	
CB	18	7	
aGVHD grade			
0-I	117	37	.54
II-IV	60	22	
cGVHD type			
None	64	28	.28
Lmt/Ext	67	20	

Correlation between the two groups was examined. Some of the missing data was not available, and total numbers do not add up to the number of the patients in each group.
RIST indicates reduced intensity stem cell transplantation; sMTX, short-course methotrexate; CyA, cyclosporin A; FK, tacrolimus; BM, bone marrow; PB, peripheral blood; CB, cord blood; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; Lmt, limited; and Ext, extensive.
*Dose of methotrexate was not surveyed in the study. Detail of other GVHD prophylaxis regimens are in Table S1.

When patients undergoing allogeneic SCT in first CR were analyzed, 3-year OS was not significantly different between patients with t(8;21) and inv(16) (84% and 74%, respectively; $P = .28$), between inv(16) and intermediate risk groups (74% and 69%, respectively; $P = .84$), or between t(8;21) and intermediate risk groups (84% and 69%, respectively; $P = .06$). However, when patients undergoing allogeneic SCT in second or third CR were analyzed, the 3-year OS of patients with inv(16) was significantly better than patients with t(8;21) (86% and 45%, respectively; $P = .008$), and better than intermediate risk patients (86% and 57%, respectively; $P = .03$). Difference was not significant between patients in the intermediate risk group and t(8;21) undergoing allogeneic SCT in second or third CR ($P = .36$). The OS of inv(16) patients undergoing allogeneic SCT not in CR was 70% at 3 years, which was also significantly better than that of t(8;21) (18%; $P = .03$) and the intermediate risk group (25%; $P = .045$).

In addition, the OS of t(8;21) undergoing allogeneic SCT in first CR was significantly better than that of the unfavorable risk group (84% and 53%, respectively; $P < .001$), but the difference between the 2 groups was not significant among patients undergoing allogeneic SCT in second or third CR. In contrast, OS was not different between inv(16) and unfavorable groups undergoing allogeneic SCT in first CR, but it was significantly different when they underwent allogeneic SCT in second or third CR (86% and 44%, for inv(16) and unfavorable groups, respectively; $P = .01$) or allogeneic SCT in non-CR (70% and 15%, respectively; $P = .006$).

Survival curves of patients who underwent autologous SCT in first CR, second or third CR, and not in CR are shown in Figure 2A, 2B, and 2C, respectively. The overall survival of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown cytogenetic risks in first CR was 77%, 59%, 74%, 38%, and 71%, respectively ($P = .049$), while that of patients undergoing autologous SCT in second or third CR was 43%, 50%, 59%, 44%, and 42%, respectively ($P = .8$). The OS of patients undergoing autologous SCT not in CR with t(8;21), inv(16), intermediate, and

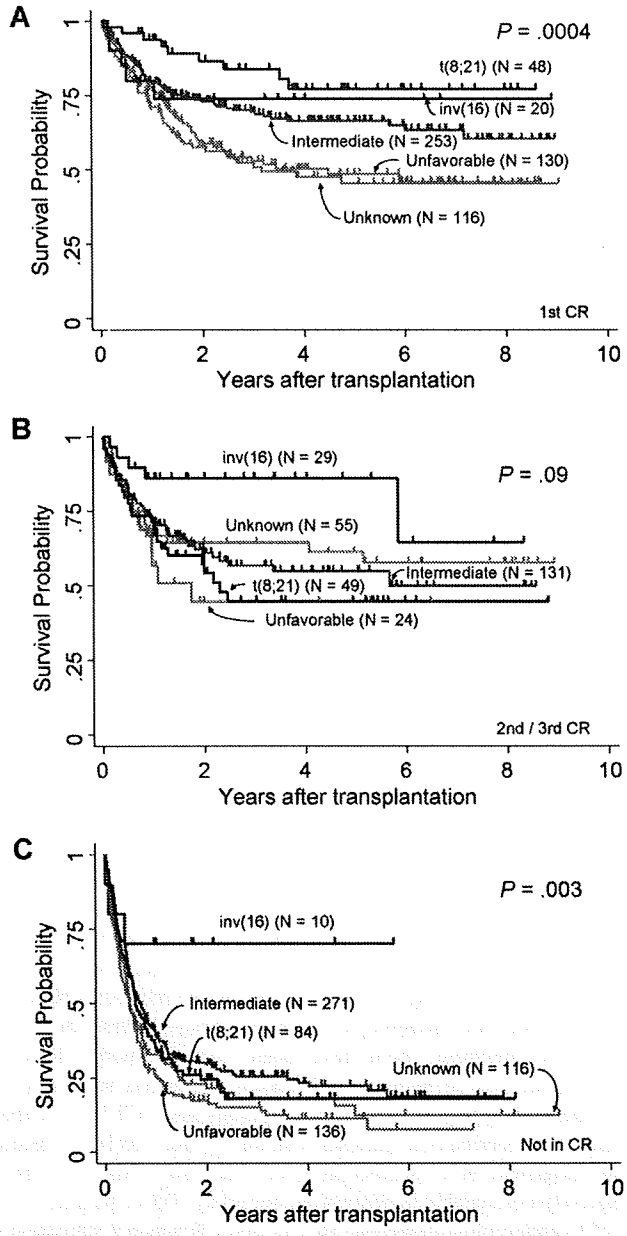

Figure 1. OS difference of patients undergoing allogeneic SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing allogeneic SCT in first CR. (B) Survival curve of patients undergoing allogeneic SCT in second or third CR. (C) Survival curves of patients undergoing allogeneic SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

unknown risks was 17%, 100%, 25%, and 13%, respectively, and the survival curve of patients in the unfavorable risk group did not reach 3 years ($P = .35$).

Figure 3A and B focus on t(8;21) and inv(16) patients, stratified according to the type of (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR). The 3-year overall survival of t(8;21) patients in first CR was not different between allogeneic and autologous transplantation (84% and 77%, respectively), as well as that of patients in second or third CR (45% and 43%, respectively) and patients not in CR (18% and 17%, respectively). Similarly, the 3-year OS of inv(16) patients was not different between allogeneic and autologous transplantation when they underwent transplantation in first CR (74% and 59%). A significant difference was observed among the 3 disease status groups of t(8;21) patients ($P < .001$; Figure 3A), but not inv(16) patients ($P = .75$; Figure 3B).

The OS of allogeneic SCT, excluding cord blood transplantation, was not different from the analysis presented here, including bone marrow, peripheral blood, and cord blood transplantation (Table S2; Figures S1,S2).

DFS after SCT was also different among cytogenetic risk groups ($P < .001$). DFS of patients with inv(16) (69% at 3 years) was better compared with t(8;21) (49%), intermediate (46%), unfavorable (31%), and unknown (41%) risk groups. Among patients undergoing allogeneic SCT in first CR, DFS was also different among cytogenetic subgroups ($P < .001$). When t(8;21), inv(16), and intermediate cytogenetic subgroups undergoing allogeneic SCT in first CR were compared, the difference was not statistically significant between t(8;21) and inv(16) (78% and 73% at 3 years; $P = .58$), between t(8;21) and intermediate risk group (78% and 63%; $P = .1$), nor between inv(16) and intermediate risk group (73% and 63%; $P = .65$). DFS of patients with t(8;21) undergoing allogeneic SCT in first CR was better than that of the unfavorable risk group (78% and 47%, respectively; $P < .001$), but the difference was not significant between inv(16) and unfavorable risk groups (73% and 47%, respectively; $P = .16$).

DFS was not significantly different when 5 cytogenetic subgroups among patients undergoing allogeneic SCT in second or third CR were compared ($P = .32$). The DFS of patients undergoing allogeneic SCT in second or third CR was not significantly different between t(8;21) and inv(16) (43% and 71% at 3 years; $P = .053$), t(8;21) and the intermediate group (43% and 47%; $P = .76$), or inv(16) and the intermediate group (71% and 47%; $P = .06$). The difference was also not significant between t(8;21) and unfavorable risk groups (43% and 42%; $P = .7$), nor between inv(16) and unfavorable risk groups (71% and 42%; $P = .06$). The DFS of patients undergoing allogeneic SCT who were not in CR was significantly different among the 5 cytogenetic subgroups ($P = .005$), and that of inv(16) (75% at 3 years) was significantly better than t(8;21) (18%; $P = .02$), the intermediate risk group (22%; $P = .03$) and the unfavorable risk group (10%; $P = .003$).

Relapse and TRM

The relapse rate (RR) after SCT also differed among cytogenetic subgroups ($P < .001$). The RR of patients with inv(16) (18% at 3 years) was lower than t(8;21) (38%), intermediate (38%), and unfavorable (56%) risk groups. The RR of t(8;21) and inv(16) after allogeneic SCT was not statistically different in either first CR (16% and 6%; $P = .45$) or second or third CR (34% and 16%, respectively; $P = .09$).

Transplantation-related mortality (TRM) of all patients with AML was 22% at 3 years. The TRM of t(8;21) (18%), inv(16) (11%), and intermediate (21%), unfavorable (24%), and unknown risk groups (27%) was significantly different among cytogenetic risk groups ($P = .02$).

Evaluation of prognostic variables in CBF

Univariate analyses of t(8;21) showed that age ($P = .004$), not in CR at transplantation ($P < .001$), allogeneic SCT ($P = .01$), and TBI regimen ($P = .006$) were significant prognostic factors indicating poor OS (Table 5). Multivariate analysis for OS revealed older age ($P = .01$) and not in CR at transplantation ($P < .001$) as the independent prognostic variables. Univariate analyses of t(8;21) patients who received allogeneic SCT in CR showed that age ($P = .02$), TBI regimen ($P = .01$), and second and third CR at

Table 4. Outcome of the AML patient population by cytogenetic risk groups

	t(8;21)		inv(16)		Intermediate		Unfavorable		Unknown		
	%	N	%	N	%	N	%	N	%	N	*P*
OS											
Allogeneic SCT											
CR1	84	48	74	20	69	253	53	130	52	116	< .001
CR2/CR3	45	49	86	29	57	131	44	24	64	55	.09
Non-CR	18	84	70	10	25	271	15	136	18	116	.003
Autologous SCT											
CR1	77	42	59	13	74	89	38	15	71	39	.05
CR2/CR3	43	7	50	2	59	15	44	6	42	18	.8
Non-CR	17	6	100	1	25	16	0	10	13	8	.35
DFS											
Allogeneic SCT											
CR1	78	48	73	19	63	249	47	129	48	113	< .001
CR2/CR3	43	48	71	27	47	129	42	22	57	54	.32
Non-CR	18	81	75	8	22	255	10	128	16	107	.005
Autologous SCT											
CR1	73	41	62	13	64	81	33	15	61	36	.09
CR2/CR3	43	7	50	2	36	14	50	6	39	18	.89
Non-CR	17	6	100	1	25	16	0	10	17	6	.45

transplantation ($P < .001$) were also significantly prognostic for poor OS. These variables remained significant after multivariate analysis. Univariate analyses for inv(16) patients showed only age ($P = .009$) to be a significant prognostic factor (Table 5). The univariate analysis of inv(16) patients who underwent allogeneic SCT in CR showed only additional karyotype abnormalities to be an unfavorable prognostic variable ($P = .009$).

Additional cytogenetic abnormalities to CBF

A total of 49 patients with t(8;21) and 14 with inv(16) had additional cytogenetic abnormalities. Data for additional cytogenetic abnormalities were obtained in 42 patients with t(8;21) and 13 patients with inv(16) (Table 6). Additional abnormalities were selected that have been reported to be prognostic by others, including loss of sex chromosome (X or Y), trisomy 8, trisomy 4, del(7q), and del(9q) for the t(8;21) group, and trisomy 22, trisomy 8, trisomy 21, del(7q), and del(9q) for the inv(16) group.[14,15,20,22,23] There were no patients with trisomy 21 in the data of patients with CBF. Patients with t(8;21) and patients with inv(16) were analyzed separately. Among t(8;21) patients undergoing allogeneic SCT, survival was not different between patients with and without additional karyotype abnormalities. When patients with inv(16) were analyzed, the survival was not different between patients with (n = 13) and without (n = 67) additional abnormalities (61% and 74%, respectively; $P = .07$). The survival of patients undergoing allogeneic SCT without additional abnormality (n = 52) was significantly better than that with additional abnormality (n = 11), (85% and 53%, respectively; $P = .004$). When analysis was restricted to patients in CR with inv(16) undergoing allogeneic SCT, a similar difference was observed (86% without additional abnormality [n = 42], and 60% with additional abnormality [n = 8], respectively; $P = .03$). Difference in OS was observed among non-CR patients with (n = 9) and without (n = 1) additional abnormality, but this difference may not be relevant with too few patients in the analysis. We further analyzed subgroups of additional abnormalities of the patients with inv(16). Although the number of patients were limited, significant difference was found among 3 groups of patients; trisomy 8 or trisomy 22 as a sole abnormality (n = 4), without additional abnormality (n = 69), and other additional abnormality to inv(16) (n = 10). The OS at 3 years were 100%, 74%, and 42%, respectively ($P = .002$). The OS of patients undergoing allogeneic SCT was also different among these 3 groups (100%, n = 3; 85%, n = 52; and 33%, respectively; $P < .001$).

Discussion

We analyzed the outcome of a large group of patients with adult CBF AML in Japan who were treated with SCT. The current study focused on the different outcome of the 2 different cytogenetic subgroups of patients with CBF AML undergoing SCT. Our study demonstrated a comparable outcome between patients with t(8;21) and inv(16) undergoing SCT in first CR, but the prognosis between these 2 cytogenetic subgroups was different beyond first CR.

In the literature, there have been several reports showing inferior survival of patients with t(8;21) compared with inv(16) patients undergoing induction chemotherapy and SCT.[14,15,20] Other studies categorized both patients with t(8;21) and inv(16) undergoing allogeneic SCT together as good-risk CBF AML,[1,21] with a relatively comparable prognosis. In our study, OS of patients with t(8;21) undergoing allogeneic SCT in first CR was not statistically different from intermediate cytogenetic subgroup (84% and 79% at 3 years, respectively; $P = .058$). Moreover, the survival of inv(16) (74% at 3 years) and intermediate cytogenetic subgroups showed no statistically significant difference.

In contrast, we have here demonstrated that the prognosis of patients with t(8;21) undergoing allogeneic SCT with second or third CR disease was significantly poor compared with those with inv(16). This finding is consistent with those of other studies reporting differences between the 2 types of CBF AML.[14,15] In the present study, non-CR disease with t(8;21) was also significantly poor compared with patients with inv(16). The Acute Leukemia French Association reported that allogeneic donor availability among patients with CBF AML who were in second CR was a prognostic factor for better survival.[16] We believe that different treatment strategies should be applied for patients with t(8;21) and those with inv(16) other than first CR.

Patients with t(8;21) undergoing allogeneic SCT and autologous SCT had a similar survival rate when they underwent transplantation in first CR, and in further CR. No survival difference between allogeneic SCT and autologous SCT was also

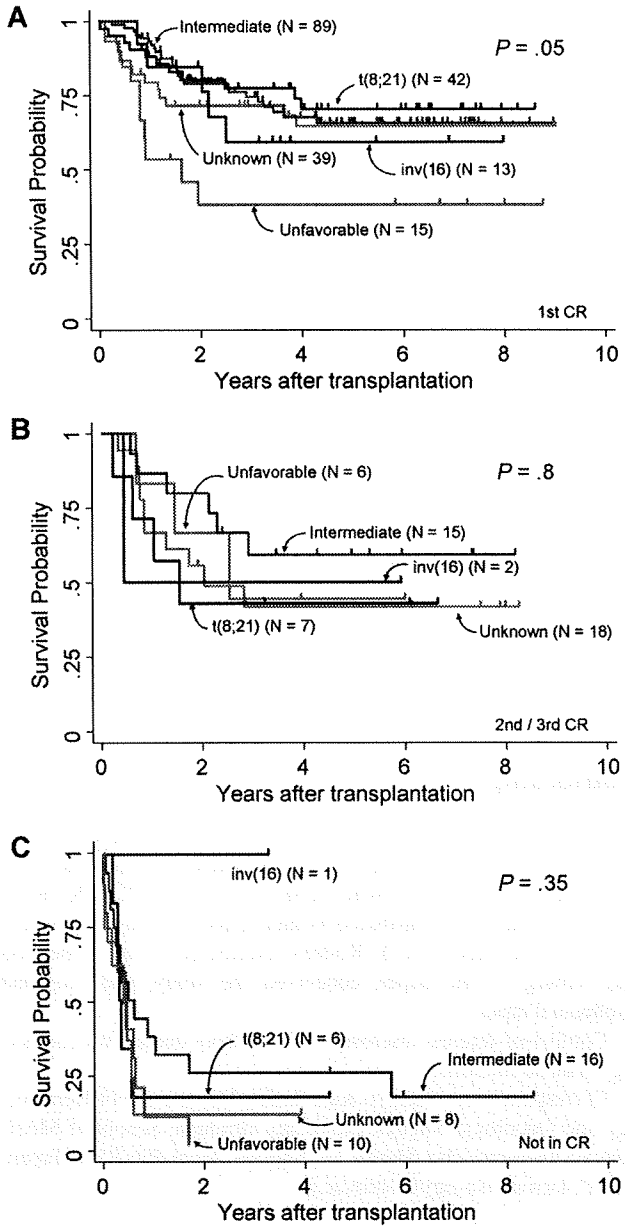

Figure 2. OS difference of patients undergoing autologous SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing autologous SCT in first CR. (B) Survival curves of patients undergoing autologous SCT in second or third CR. (C) Survival curves of patients undergoing autologous SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

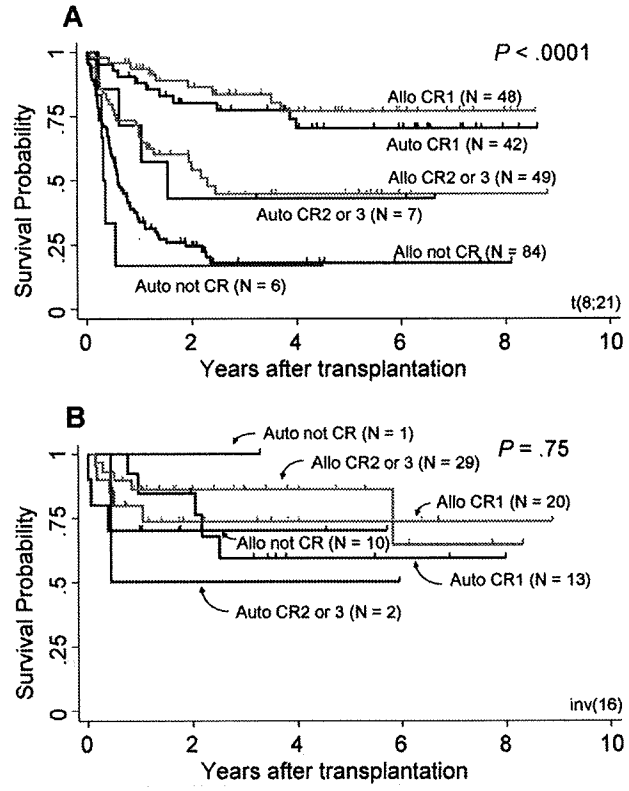

Figure 3. OS of patients with CBF. Survival curves of patients with t(8;21) (A) and with inv(16) (B). Both are stratified according to the type of transplantation (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR).

observed among inv(16) patients receiving SCT in first CR (74% and 59%, respectively). The University of California, San Francisco (UCSF) group described the good results of patients with advanced AML undergoing autologous SCT in second or third remission, including patients with CBF.[24] As in our study, the European Group for Blood and Marrow Transplantation (EBMT) reported that the survival rate of t(8;21) patients who received allogeneic bone marrow transplantation was not significantly different from that of patients who received autologous SCT.[1] Results by others showed that allogeneic SCT in first CR did not benefit good-risk cytogenetic subgroups.[3,25,26] Schlenk et al also demonstrated that t(8;21) patients receiving allogeneic SCT or chemotherapy showed no difference in outcome.[23] These results suggest that autologous SCT can be considered as postremission therapy for patients with CBF AML, but it remains unclear whether SCT is more beneficial for patients with CBF than high-dose cytarabine. Survival of patients with inv(16) was favorable beyond first CR. Patients with inv(16) in second or third CR, or even non-CR patients, are good candidates for allogeneic SCT. There are long-term survivors after allogeneic SCT in non-CR disease, so t(8;21) patients with no other choice of treatment, such as those in further CR or non-CR, can proceed to allogeneic SCT. In order to confirm the appropriate treatment for t(8;21) patients in first CR, a prospective trial is needed to compare the results of autologous SCT for t(8;21) in first CR with standard chemotherapy. t(8,21) patients with suitable related or well-matched donors should be recommended to participate in a risk-adopted prospective trial when they receive allogeneic SCT in first CR.

There were differences between the 2 types of CBF AML with respect to prognostic valuables. Age was a significant and independent prognostic variable in both t(8;21) and inv(16) patients, a finding in agreement with reports from some,[14,27] but not all,

Table 5. Prognostic factors affecting overall survival of patients with t(8;21)

Variables	Unfavorable factors	Hazard ratio	95% CI	P
t(8;21)				
Age		1.02	1.01-1.04	.004
Disease status at SCT	Not in CR	4.4	3.1-6.5	< .001
Transplantation	Allo-SCT	1.9	1.2-3.0	.01
Conditioning regimen	TBI	1.7	1.2-2.5	.005
inv(16)				
Age		1.1	1.0-1.1	.009

CI indicates confidence interval.