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Clinical Significance of Serum Hepcidin Levels on Early Infectious Complications in Allogeneic Hematopoietic Stem Cell Transplantation

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The association of iron overload with complications of allogeneic hematopoietic stem cell transplantation (HSCT) has been suggested in previous studies. Because hepcidin plays a central role in the regulation of iron homeostasis, we analyzed the association between pretransplant serum hepcidin-25 levels and early infectious complications after allogeneic HSCT. We studied 55 consecutive adult patients with a median age of 47 years (range: 20–64 years) who underwent allogeneic HSCT for hematologic malignancies at our institution. Thirty-two patients had myelogenous malignancies; the remaining 23 had lymphogenous malignancies. The median pretransplant serum hepcidin level of patients in the study was 21.6 ng/mL (range: 1.4–371 ng/mL), which was comparable to that of healthy volunteers (median: 19.1 ng/mL [range: 2.3–37 ng/mL]; n = 17). When cumulative incidences of documented bacterial and cytomegalovirus (CMV) infections at day 100 were compared according to pretransplant hepcidin-25 levels, the incidence of bacterial, but not CMV, infection, was significantly higher in the high-hepcidin group (≥50 ng/mL; n = 17) than in the low-hepcidin group (<50 ng/mL; n = 38) (65% [95% confidence interval, 38%–82%] versus 11% [3%–23%]; $P < .001$). This finding was confirmed by multivariate Cox analysis adjusted for confounders, including pretransplant ferritin and C-reactive protein (CRP) levels. No fungal infection was documented in either group. These results suggest that the pretransplant serum hepcidin-25 level may be a useful marker for predicting the risk of early bacterial complications after allogeneic HSCT. Larger prospective studies are, however, warranted to confirm our findings.

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has been widely performed as a potentially curative treatment for intractable hematologic malignancies with conventional chemotherapy. However, despite recent advances in the treatment of infectious diseases and conditioning regimens for transplantation, treatment-related complications remain a major problem. Therefore, it is particularly important to identify a good biomarker that can predict treatment-related complications before transplantation. A recently accumulated body of evidence suggests that iron overload is associated with adverse clinical outcomes in HSCT [1-10]. Armand et al. [2] showed that a high pretransplant serum ferritin level was strongly associated with lower overall and disease-free survival (OS, DFS) in patients with allogeneic HSCT that was performed as a treatment for acute leukemia and myelodysplastic syndrome (MDS). Other studies have shown that pretransplant iron overload in autologous or allogeneic HSCT was a risk factor associated with posttransplant complications, such as mucositis, bacterial, and fungal infection, and hepatic veno-occlusive disease (VOD) [3-6,8-11].

Hepcidin, first identified in human blood and urine as an antimicrobial small peptide [12,13], is now considered to be a central molecule that regulates iron metabolism. Hepcidin decreases iron absorption from the intestine and blocks its release from iron stores by

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downregulating the expression of the cellular iron exporter, ferroportin [14,15]. Hepatic expression of hepcidin can be upregulated by iron loading [16,17] as well as by inflammatory stimuli such as interleukin-6 (IL-6) [18]. Therefore, we hypothesized that serum hepcidin level could be a useful predictor of iron overload and inflammatory condition prior to HSCT. Here, we performed a single-center retrospective study at our institution to evaluate the significance of serum hepcidin levels as a predictor of early treatment-related complications after allogeneic HSCT with special reference to infectious complications.

PATIENTS AND METHODS

Study Population

The study population comprised 66 consecutive adult patients who underwent allogeneic HSCT for the treatment of hematologic malignancies at Kyoto University Hospital from July 2006 to September 2008. A total of 55 patients, excluding those who had received prior transplantations within 1 year or who had any active infections before the current transplantation, were included in the analysis. This study was approved by the Ethics Committee of Kyoto University Graduate School and the Faculty of Medicine. Written informed consent was obtained from all patients.

Serum Analysis

Before the administration of conditioning regimens, serum samples were obtained at around 8:00 am, allocated in tubes, and stored at −80°C until analysis. The levels of serum hepcidin-25 (the main form of active hepcidin peptide) were quantified using a liquid chromatography-tandem mass spectrometry-based assay system following the method described by Murao et al. [19]. Other serum parameters were measured using standard laboratory techniques.

Prophylaxis, Monitoring, and Diagnosis of Infection

The patients were isolated in a single room equipped with a high-efficiency particulate air filter (HEPA) system from 1 day before transplantation until at least 4 weeks after transplantation. No bacterial prophylaxis was prescribed for the patients according to our institutional protocols [20]. Trimethoprim-sulfamethoxazole (160 mg/day [trimethoprim], 3 times a week) was administered as prophylactic therapy for *Pneumocystis jirovecii* pneumonia from the day of admission until the day of transplantation and restarted after the day of neutrophil engraftment. All patients received fluconazole (200 or 400 mg/day) and acyclovir (1000 mg/day) prophylaxis from the period of conditioning until 30 days after transplantation. After the first 30 days, the patients received fluconazole at a dose of 100 mg/day until at least 100 days after transplantation. The administration of acyclovir (400 mg/day) was continued when patients received steroid therapy for acute graft-versus-host disease (aGVHD). For each febrile episode, 1 or 2 sets of blood samples were cultured, and the cultures of specimens other than blood and imaging examinations were performed according to clinical judgment. The occurrence of cytomegalovirus (CMV) infection was closely monitored by CMV pp65 antigenemia testing with C10/C11 monoclonal antibodies (mAbs) from the day after neutrophil engraftment until at least 100 days after transplantation. Documented bacterial infection included any incidence of bloodstream infection or any other bacterial infection. Bloodstream infection was diagnosed if at least 1 of the following criteria was met: (1) blood culture obtained during a febrile episode was positive, at least once, for bacterial organisms not considered to be common skin contaminants; (2) blood culture obtained during a febrile episode was positive for the same common skin contaminant on separate occasions within 72 hours; (3) blood culture was positive, at least once, for a common skin contaminant, and the patient was diagnosed with septicemia, including hypotension (systolic blood pressure, <90 mmHg) and abnormal coagulopathy. Infections other than bloodstream infection were diagnosed if the following criteria were met: (1) bacterial organisms were observed from specimens such as sputum, urine, and stool at least on 2 occasions, and (2) the patient showed symptoms of infection corresponding to those specimens. *Clositridium difficile* enterocolitis was excluded from the analysis, because this disease is toxin-mediated, and cannot be prevented by administration of common bacterial prophylactic agents such as fluoroquinolones, even if patients with a high risk of bacterial infection can be identified by using a putative biomarker. CMV infection was defined as positive if either C10 or C11 antigenemia assay showed at least 2 positive cells per 150,000 leukocytes. Invasive fungal infection was diagnosed according to the criteria of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group [21].

Statistical Analysis

Endpoints included cumulative incidences of documented bacterial infection, fungal infection, CMV infection, and infection-related mortality, and OS within 100 days post transplantation. Patient and transplant characteristics between 2 groups were compared using the Mann-Whitney *U*-test or χ^2 analysis, as appropriate. The day of neutrophil

engraftment was defined as the first of 3 consecutive days when the absolute neutrophil count (ANC) exceeded 500/μL. The day of neutrophil engraftment between 2 groups was compared by using the Mann-Whitney *U*-test. To eliminate the effect of competing risk, the cumulative incidences were assessed using methods described elsewhere [22]. The competing event in the cumulative incidence analyses was defined as death without an event of interest within 100 days post transplantation. OS was estimated using Kaplan-Meier methods. Infection-related death was defined as death associated with any infection within 100 days after transplantation. Standard risk disease was defined as complete remission (CR) in cases of acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), adult T cell leukemia/lymphoma (ATL), Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), and untreated or CR in MDS and myeloproliferative disorder (MPD). High-risk disease was defined as statuses other than CR in AML, ALL, ATL, HL, and NHL and in MDS and MPD after treatment. The Cox proportional-hazard model was applied to assess the effect of factors that potentially affected the study endpoints. The following items were added as confounders: recipient's sex (male or female), recipient's age (<50 or ≥50 years), diagnosis (myelogenous or lymphogenous malignancies), risk of disease (standard or high risk), conditioning regimen (reduced or myeloablative intensity [RIC, MA]), type of donor (related or unrelated donor), reticulocyte count ($<60 \times 10^9$ or $\geq 60 \times 10^9$/L), ferritin level (<1000 or ≥1000 mg/dL), and C-reactive protein (CRP) level (<0.3 or ≥0.3 μg/dL). The cutoff points for reticulocyte count and the ferritin and CRP levels were chosen such that we could make optimal use of the information with a proviso that the smaller group contained at least 30% of patients. *P* values of < .05 were considered statistically significant. All analyses were conducted using STATA software version 10 (STATA Corp., College Station, TX).

Table 1. Characteristics of Patients and Transplants

Variables	Hepcidin, Low (<50 ng/mL) n = 38	Hepcidin, High (≥50 ng/mL) n = 17	*P* Value
Age at transplant			
Median age (range)	47.5 (23–64)	47 (20–63)	.750
Sex			.171
Male	21 (55%)	6 (35%)	
Female	17 (45%)	11 (65%)	
Disease			.612
Myeloid malignancies	23 (61%)	9 (53%)	
AML	15	8	
MDS/MPD	8	1	
Lymphoid malignancies	15 (39%)	8 (47%)	
ALL	4	4	
ATL	4	1	
HL	1	0	
NHL	6	3	
Risk of disease			.051
Standard	22 (58%)	5 (29%)	
High	16 (42%)	12 (71%)	
Conditioning regimen			.545
Myeloablative intensity	19 (50%)	10 (59%)	
Reduced intensity	19 (50%)	7 (41%)	
Prophylaxis against GVHD			.663
Cyclosporine-based	5 (13%)	3 (18%)	
Tacrolimus-based	33 (87%)	14 (82%)	
Type of donor			.181
Related donor			
HLA*-matched	10 (26%)	3 (18%)	
HLA-mismatched	3 (8%)	1 (6%)	
Unrelated donor			
HLA-matched	18 (47%)	5 (29%)	
HLA-mismatched	7 (18%)	8 (47%)	
Source of stem cells			.259
Bone marrow	29 (76%)	10 (59%)	
Peripheral blood	1 (3%)	0 (0%)	
Cord blood	8 (21%)	7 (41%)	
Serum ferritin (μg/dL) mean (±SD)	664 (±796)	1551 (±993)	<.001
CRP (mg/dL) mean (±SD)	0.36 (±0.68)	0.70 (±1.63)	.176
Reticulocyte ($\times 10^9$/L) mean (±SD)	63.7 (±40.2)	64.0 (±42.2)	.979

AML indicates acute myelogenous leukemia; MDS/MPD, myelodysplastic syndrome and myeloproliferative disorders; ALL, acute lymphoblastic leukemia; ATL, acute T cell leukemia/lymphoma; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; GVHD, graft-versus-host disease; Cyclosporine-based, cyclosporine with or without other agents; Tacrolimus-based, tacrolimus with or without other agents; HLA, human leukocyte antigen; CRP, C-reactive protein.
Data are counts of individuals unless specified otherwise.
*HLA compatibility was defined according to the results of serologic or low-resolution molecular typing for HLA-A, -B, and -DR antigens.

RESULTS

Characteristics of Patients and Transplants

Characteristics of patients and transplants are shown in Table 1. The median age of patients was 47 years (range: 20–64 years). The primary disease in these patients was as follows: AML in 23, MDS/MPD in 9, ALL in 8, NHL in 9, HL in 1, and ATL in 5. The risk of diseases was standard in 27 and high in 28 patients. Nearly half of the patients (n = 26) received a RIC regimen. The stem cell sources used were bone marrow (BM) in 39, peripheral blood (PB) in 1, and cord blood (CB) in 15 patients. The median pretransplant serum hepcidin level was 21.6 ng/mL (range: 1.4–371 ng/mL), which was comparable to that of healthy volunteers (median: 19.1 ng/mL [range: 2.3–37 ng/mL]; n = 17) [23]. Because the lower hepcidin level of the third tertile among the patients in this study was 49.1 ng/mL, we set a cutoff hepcidin level of 50 ng/mL for practical use to divide the patients into low- and high-hepcidin groups (n = 17 and 38, respectively). There was no difference in patient and transplant characteristics between the low- and high-hepcidin groups, except for serum ferritin levels ($P < .001$).

Documented Bacterial Infections

There was no significant difference between the days of neutrophil engraftment of the low- and high-hepcidin groups (median day: 21 [range: 14–99] and median day: 22.5 [range: 12–53], respectively, $P = .54$). A total of 16 episodes of bacterial infections were documented; these included 15 episodes of bloodstream infections and 1 episode of pneumonia. No patient experienced more than 1 episode of bacterial infection within 100 days after transplantation. The documented bacterial organisms are listed in Table 2. The main organisms were Gram-negative bacilli in both the low- and high-hepcidin groups. In the antimicrobial-susceptibility tests, 12 of the 13 Gram-negative isolates were sensitive to fluoroquinolone. We documented 2 bacterial infections in the late period of transplantation; 1 patient showed infection at day 89 after transplantation, which was attributed to delayed neutrophil engraftment, and another patient showed infection at day 68, when the neutrophil counts had temporarily decreased. The cumulative incidences of the documented bacterial infection in the low- and high-hepcidin groups were 11% (95% confidence interval [CI], 3%–23%) and 65% (95% CI, 38%–82%), respectively (Figure 1A). In the low-hepcidin group, the cumulative incidence of bacterial infection was lower in patients with a hepcidin level of <25 ng/mL than in those with a hepcidin level ranging from ≥25 to <50 ng/mL (10% [95% CI, 2%–23%] versus 17%, [95% CI, 1%–52%]). Univariate analysis of various potential confounders showed that high hepcidin level was the only factor that affected the cumulative incidence of documented bacterial infection (hazard ratio [HR], 8.98; 95% CI, 2.82–28.57; $P < .001$) (Table 3). To exclude the effect of other confounders, the significance of high hepcidin level was assessed in the stratified category of each confounder (eg, in either the high- or low-ferritin group); we noted consistently high HRs in the high-hepcidin group in each stratified category (data not shown). We also found that hepcidin had a significant impact on the patients, excluding the patients in other specific categories, such as those who received a CB transplant or those who underwent a transplant from an unrelated HLA-mismatched donor (data not shown). Furthermore, the significant effect of hepcidin persisted even after the adjustment for confounders in multivariate analysis (HR, 28.46; 95% CI, 2.51–323.34; $P = .007$) (Table 3). Even when the variables were treated as continuous instead of categoric, the significant effect of hepcidin persisted (HR, 1.01; 95% CI, 1.00-1.01; $P = .001$).

Table 2. Documented Bacterial Organisms within 100 Days after Stem Cell Transplantations

Category	Hepcidin, Low (<50 ng/mL) n = 38	Hepcidin, High (≥50 ng/mL) n = 17
Gram-positive cocci (n)	*Staphylococcus epidermidis* (1)	*Enterococcus faecium* (2)
Gram-negative bacilli (n)	*Klebsiella pneumoniae* (2)	*Klebsiella pneumoniae* (3)
	Enterobacter cloacae (1)	*Escherichia coli* (3)
	Prevotella intermedia (1)	*Pseudomonas aeruginosa* (2)
		Klebsiella oxytoca (1)

P. intermedia was detected in the sputum of 1 patient with pneumonia. Other organisms were detected in blood culture bottles.

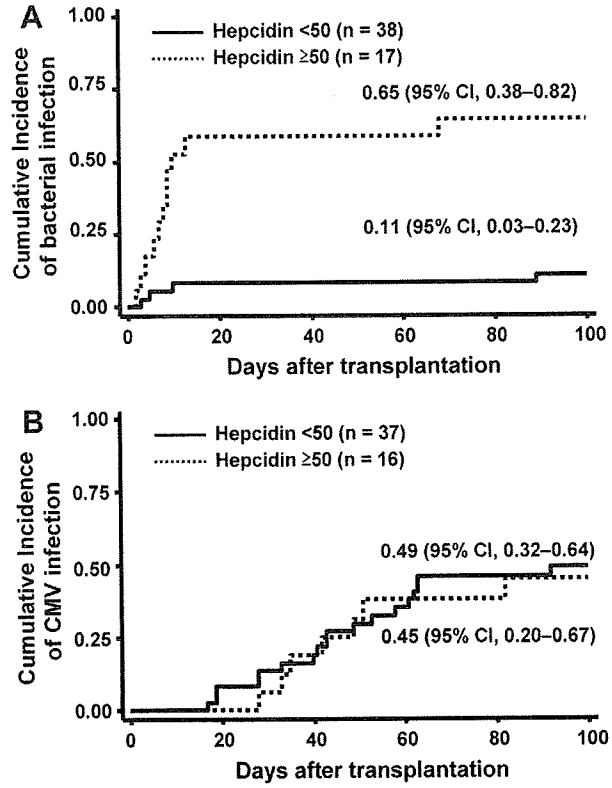

Figure 1. The cumulative incidences of documented bacterial infection (A) and cytomegalovirus (CMV) infection (B) at 100 days after stem cell transplantation. Solid black line, the low-hepcidin group (<50 ng/mL); solid gray line, the high-hepcidin group (≥50 ng/mL); CI, confidence interval. CMV infection was not assessable in 2 patients because of early death before neutrophil engraftment.

Other Transplant-Related Complications and Mortality

The cumulative incidences of CMV infection in the low- and high-hepcidin group were 49% (95% CI, 32%–64%) and 45% (95% CI, 20%–67%), respectively (Figure 1B); univariate and multivariate analyses showed no significant difference between the 2 groups (Table 3). All CMV infections were well treated by the administration of ganciclovir or foscarnet. No fungal infection was documented. Therefore, all infection-related deaths were attributed to bacterial infection. The cumulative incidence of infection-related mortality in the low-hepcidin group was 3% (95% CI, 0.2%–12%), whereas that in the high-hepcidin group was 6% (95% CI, 0.4%–24%),

Table 3. Univariate and Multivariate Analyses of Documented Bacterial Infection, CMV Infection, and Overall Survival at 100 Days after Stem Cell Transplantations

		Univariate Analysis		Multivariate Analysis	
	Number	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
1) Documented bacterial infection					
Hepcidin, low (<50 ng/mL)	5/38	1	–	1	–
Hepcidin, high (≥50 ng/mL)	11/17	8.98 (2.82–28.57)	<.001	28.46 (2.51–323.34)	.007
2) CMV antigenemia (C10 or C11 ≥2)					
Hepcidin, low (<50 ng/mL)	18/37	1	–	1	–
Hepcidin, high (≥50 ng/mL)	7/16	0.97 (0.40–2.32)	.939	0.63 (0.16–2.49)	.511
3) Overall survival					
Hepcidin, low (<50 ng/mL)	36/38	1	–	–	–
Hepcidin, high (≥50 ng/mL)	14/17	3.60 (0.60–21.56)	.161	–	–

CMV indicates cytomegalovirus; CI, confidence interval.
Hazard ratios (HRs) in multivariate analysis were adjusted for recipient's sex (male or female), recipient's age (<50 or ≥50 years), diagnosis (myelogenous or lymphoid malignancies), risk of disease (standard or high risk), conditioning regimen (reduced or myeloablative intensity), type of donor (related or unrelated donor), reticulocyte count (<60 × 109 or ≥60 × 10^9/L), ferritin level (<1000 or ≥1000 mg/dL), and C-reactive protein (CRP) level (<0.3 or ≥0.3 μg/dL). Overall survival was not analyzed in the multivariate model because of the low incidence of death.

with no statistical difference between the 2 groups. OS at 100 days after transplantation in the low- and high-hepcidin groups was 95% (95% CI, 81%–99%) and 82% (95% CI, 55%–94%), respectively (Figure 2). No significant difference in OS was observed (Table 3).

DISCUSSION

In our cohort of patients who underwent allogeneic HSCT for hematologic malignancies, we found a significant association between the pretransplant serum hepcidin levels and the cumulative incidence of documented bacterial infection. To our knowledge, this is the first study that has evaluated the clinical significance of serum hepcidin levels in predicting transplant-related complications; the findings suggest that the pretransplant serum hepcidin level can be used as a good pretransplant biomarker to predict bacterial infection in a patient scheduled for HSCT.

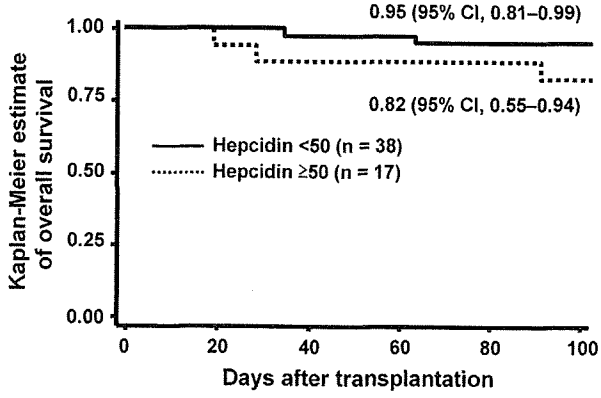

Figure 2. Kaplan-Meier estimate of OS at 100 days after stem cell transplantation. Solid black line, the low-hepcidin group (<50 ng/mL); solid gray line, the high-hepcidin group (≥50 ng/mL); CI, confidence interval.

Hepcidin production is regulated by at least 3 factors: iron load [16,17], inflammation [18], and unknown erythropoietic signals [23-25]. Therefore, the good predictive value of hepcidin with respect to the incidence of documented bacterial infection can be partly explained by the cumulative effect of at least these 3 factors on bacterial infection. Iron overload increases the level of circulating non-transferrin-bound iron, which is known to amplify free-radical reactions in inflammatory or ischemia-related conditions [7,26]. Such reactions could enhance tissue damage such as mucositis during the conditioning regimen, thereby allowing bacterial translocation through the damaged mucosa [27]. In addition, iron is a necessary nutrient for bacteria and fungus [28]. The association between hemochromatosis, 1 of the iron overload disorders, and infection with certain organisms has already been described [29]. Therefore, the high hepcidin levels might reflect iron overload status, which has an adverse effect on bacterial infections. Second, a high hepcidin level may indicate inflammation because of a latent bacterial infection that was undetectable before HSCT, but may surface in posttransplant neutropenic status. Last, a high hepcidin level could reflect suppressed erythropoiesis, probably because of the short duration from the last chemotherapy to the start of the conditioning regimen for transplantation. Repeated cytotoxic chemotherapy in a short period may exacerbate tissue damage and increase the risk of bacterial infection.

Although serum ferritin levels do not necessarily correlate with the amount of iron load in patients with inflammation or specific diseases [1,30,31], it is frequently used and regarded as a indicator of iron overloading, and several studies have demonstrated the association between high ferritin levels and treatment-related mortality (TRM) [3,11]. In this cohort, an elevation of serum ferritin level was not found to be a significant risk factor for bacterial infection,

whereas an elevated hepcidin level was a strong risk factor even after adjustment for other potential confounders. Furthermore, we observed consistent association of high hepcidin levels with high risk for developing bacterial infection when analyses were confined to either the low- or high-ferritin subgroups. These findings collectively suggest that hepcidin can be used as a better predictor of documented bacterial infections than serum ferritin levels. Moreover, various new techniques to quantify hepcidin-25, such as a competitive enzyme-linked immunoassay as well as mass spectrometry-based methods, have been recently developed [19,25,32,33]. Standardization of those methods will make it possible to use the serum hepcidin level as a biomarker in routine clinical practice.

Hepcidin was first isolated and characterized as an antimicrobial peptide in human blood [12]. In radial diffusion assays, synthetic hepcidin suppressed the growth of several strains of Gram-positive bacteria and some strains of Gram-negative bacteria, but not of *Escherichia coli* or *Pseudomonas fluorescens*. Our findings pertaining to the adverse association of high hepcidin levels with bacterial infection indicated that the bactericidal effect of hepcidin was either considerably limited in neutropenic settings such as HSCT or was ineffective on the bacterial organisms observed in our cohort. Moreover, we observed a significant adverse effect of hepcidin even after the adjustment for potential confounders, suggesting that hepcidin itself may play an unknown biologic role in susceptibility to bacterial infection, or it may represent an unknown surrogate marker for predicting bacterial infection. To answer this issue, the significance of pretransplant serum hepcidin levels needs to be evaluated in a more homogeneous group of patients having the same level of confounders.

We did not detect any adverse effect of high hepcidin levels on infection-related mortality or OS at 100 days after transplantation, although there was a marked difference in the incidence of bacterial infection. One possible explanation for this observation is that bacterial infection of the blood was well managed by prompt and appropriate treatment with antibiotics in our transplant centers. However, because the incidence of early death after HSCT is considerably low, the effect of bacterial infection on early mortality should be evaluated in larger cohort studies to gain enough statistical power for comparison. Alternatively, selective prophylactic administration of oral antibiotics such as fluoroquinolones to patients with a high risk of bacterial infection may be an effective approach; however, this approach will be effective only if most of the bacterial isolates at the transplant center are sufficiently sensitive to these prophylactic antibiotics. With regard to other endpoints, there was no association between high hepcidin levels and the incidence of CMV infection. The effect of hepcidin level on the incidence of fungal infection could not be evaluated because of the very low incidences of these conditions in our cohorts. These effects should also be evaluated in studies with a larger cohort in the future.

The present study, however, has some limitations. We cannot exclude the possibility of a pseudonegative result for bloodstream infection, because broad-spectrum antibiotics were administered to all neutropenic patients at the time of blood culture, regardless of the results of blood culture. In addition, the retrospective study design and heterogeneous background of diseases and transplantation procedures could also bias the results. Particularly, in the small cohort of 55 patients, the adjustment of HRs by confounders may be incomplete. In particular, the higher proportion of CB transplants and the high risk of diseases in the high-hepcidin group may cause bias, although we found consistently high HRs in the high-hepcidin group in various stratified categories. Therefore, larger studies are necessary to confirm our results.

In conclusion, our study revealed that the pretransplant serum hepcidin level was significantly associated with bacterial infection, particularly bloodstream infection, suggesting that quantification of serum hepcidin levels could be useful for predicting early bacterial complications. Prophylactic antibiotic therapy based on the local sensitivities of common bacterial isolates can be considered in the patients with high hepcidin levels who are undergoing allogeneic HSCT. Larger prospective studies are, however, warranted to confirm our findings.

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Conflict of interest: N.T. declares that he is the President of Medical Care Proteomics Biotechnology Co. Ltd. (Ishikawa-ken, Japan), a startup company, the stock of which is not publicly traded. The other authors declare that they have no conflicts of interest relevant to this paper.

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ORIGINAL ARTICLE

Mycophenolate mofetil combined with tacrolimus and minidose methotrexate after unrelated donor bone marrow transplantation with reduced-intensity conditioning

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Abstract We evaluated the efficacy of a post-grafting immunosuppressive regimen consisting of tacrolimus, methotrexate, and mycophenolate mofetil (MMF) in 21 adults (median age, 55 years) with poor-risk hematologic malignancy who underwent unrelated bone marrow transplantation after fludarabine-based reduced-intensity conditioning (RIC). In combination with intravenous tacrolimus and minidose methotrexate (5 mg/m^2 on days 1, 3, and 6), MMF was orally administered at 30 mg/kg daily in three divided doses between days 7 and 27. All patients achieved neutrophil recovery with donor-type chimerism at a median of 19 days (range, 13–35). Cumulative incidences of grades II–IV and III–IV acute graft-versus-host disease (GVHD) were 33% (95% CI, 15–53%) and 5% (95% CI, 0.3–20%), respectively. Five of 20 evaluable patients developed extensive chronic GVHD. Toxicities associated with the use of MMF were acceptable, although one patient experienced intractable GVHD immediately after the cessation of MMF. With a median follow-up of 24 months, overall survival at 3 years was 38% (95% CI, 14–63%). No late graft failure was observed. In conclusion, post-transplant MMF combined with tacrolimus and methotrexate was well tolerated and conferred stable donor cell engraftment, low risk of severe acute GVHD, and encouraging overall survival in unrelated donor marrow transplantation after RIC regimens.

Keywords Mycophenolate mofetil · Reduced-intensity conditioning · Unrelated donor · Bone marrow transplantation · Graft-versus-host disease

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) with reduced-intensity conditioning (RIC) regimens is increasingly employed as a treatment option for various hematologic disorders. RIC transplantations using cytokine-mobilized peripheral blood stem cells (PBSC) have been reported to yield comparable outcomes with conventional myeloablative HSCT at least in selected patients [1–4]. However, previous reports have consistently shown that RIC transplantations using bone marrow (BM) graft, especially from an unrelated donor, are associated with an increased risk of graft failure and treatment-related toxicity as compared with those using PBSC, although confirmatory data from randomized-controlled trials are currently unavailable [5–8]. To improve outcomes after unrelated BM transplantation conditioned with non-myeloablative or reduced-intensity regimens, it would be beneficial to introduce a newer post-transplant immunosuppressive protocol which can effectively prevent both graft rejection and severe graft-versus-host disease (GVHD).

Mycophenolate mofetil (MMF) is an esterified prodrug of mycophenolic acid (MPA), which has pleiotropic immunosuppressive actions [9, 10]. MPA preferentially inhibits de novo purine nucleotide synthesis in T-cells and B-cells via inhibition of inosine-5′-monophosphate

dehydrogenase, interfering with their proliferation. MPA also suppresses dendritic cell maturation and can induce T-cell apoptosis. The use of MMF in combination with cyclosporine or tacrolimus was proven to be active in promoting hematopoietic stem cell engraftment after non-myeloablative HSCT using fludarabine and low-dose total-body irradiation (TBI) conditioning [11, 12], and was also shown to be as effective as the standard post-grafting immunosuppression with cyclosporine and methotrexate (MTX) in preventing severe acute GVHD after myeloablative HSCT from an HLA-matched related donor [13, 14].

A combination of tacrolimus and minidose MTX has been widely used as GVHD prophylaxis in RIC transplantation as well as in conventional HSCT from adult unrelated donors [15, 16]. Because MMF and tacrolimus are shown to have synergistic immunosuppressive actions in experimental and clinical organ transplantations [17, 18], we hypothesized that MMF in conjunction with tacrolimus and minidose MTX would be more efficacious than a combination of tacrolimus/MTX alone. In this single-center study, we retrospectively evaluated the efficacy of such triple combination as an alternative peritransplant immunosuppressive protocol in unrelated donor RIC transplantation using exclusively BM as a stem cell source.

2 Patients and methods

2.1 Patients

Among 61 consecutive adult patients who received BM transplantation from an unrelated donor between 2003 and 2006 at Kyoto University Hospital, those who fulfilled the following criteria were selected for the study: having a hematologic malignant disease; having an unrelated donor who was serologically matched at HLA-A, -B, and -DR antigens, allowing a single-allele mismatch identified by high-resolution DNA typing; receiving fludarabine-based RIC because of having a history of chemoradiotherapy precluding the use of myeloablative conditioning or having 55 through 69 years of age; receiving GVHD prophylaxis consisted of intravenous tacrolimus, minidose MTX and oral MMF. All patients had an adequate cardiac, pulmonary, hepatic, and renal function at the time of transplantation and did not have therapy-resistant central nervous system involvement or active infectious disease. A total of 21 patients fulfilled these criteria and considered evaluable for the study. With respect to disease status at transplant, patients who received transplant without prior cytotoxic chemotherapy or in first complete remission were considered to have an early disease, while those who underwent transplantation in all the other conditions were considered to have an advanced disease. All the patients with early disease were considered to have resistance to conventional chemotherapy or to have a high risk of relapse: those included two cases with untreated high-risk myelodysplastic syndrome, one with chronic active Epstein-Barr virus infection and one with adult T-cell leukemia/lymphoma in first remission. This study was approved by the Institutional Review Board and Ethic Committee of Kyoto University; written informed consent for transplantation was obtained from all participating patients.

2.2 Study end points

The primary end points of the study were donor cell engraftment and the occurrence of grade II–IV acute GVHD. Secondary end points included the neutrophil and platelet recovery, the occurrence of extensive chronic GVHD, progression or relapse of primary disease, and death from any cause.

Donor cell engraftment was defined as the detection of donor-type chimerism among unfractionated BM-nucleated cells with concomitant neutrophil recovery. Date of neutrophil recovery was defined as the first 3 consecutive days with the absolute neutrophil count (ANC) higher than 0.5×10^9/L. Date of platelet recovery was defined as the first 7 consecutive days with platelet count exceeding 20×10^9/L without transfusion. Acute GVHD was diagnosed and graded according to the conventional criteria [19]. Chronic GVHD was diagnosed and staged as limited or extensive on the basis of traditional criteria among patients who survived more than 90 days after transplantation [20]. Disease response and progression were defined by the standard criteria [21–25]. Toxicity observed between days 0 and 100 after transplantation was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver 3.0. Non-infectious pulmonary complications were diagnosed on the basis of clinical manifestations, radiologic findings, and the results of pulmonary function tests if available [26].

2.3 HLA typing and chimerism analysis

Compatibility at *HLA-A*, *-B*, and *-DRB1* loci between patients and donors was determined by standard serologic technique and high-resolution DNA typing as described elsewhere [27]. *HLA-C* compatibility was not included as a criterion for donor selection because routine *HLA-C* allele typing for the screening of unrelated donors was not available before April 2004. Donor cell chimerism levels among unfractionated BM-nucleated cells were evaluated on day 28 and thereafter at the appropriate time point by polymerase chain reaction-based analysis of polymorphic microsatellite regions for recipients of sex-matched graft or fluorescent in situ hybridization analysis of sex chromosomes for sex-mismatched pairs as described previously [28].

2.4 Transplantation procedure

Preparative regimens were assigned according to diagnosis and disease status at transplantation. Fourteen patients received fludarabine 25 mg/m^2/day for 5 consecutive days (days −6 to −2) in combination with oral busulfan 1 mg/kg every 6 h for 2 days (days −3 and −2) followed by 400 cGy of TBI in 2 fractions (on day −1 and/or day 0). One patient who had a history of TBI-based myeloablative allogeneic transplantation received the same dose schedule of fludarabine plus busulfan regimen without 400 cGy TBI. Four patients received fludarabine at the same daily dose from days −8 through −4 combined with melphalan 70 mg/m^2/day on days −3 and −2. The remaining two patients without a history of cytotoxic chemotherapy received 200 cGy TBI in a single fraction in addition to the fludarabine plus melphalan regimen.

All BM collections from unrelated donors were facilitated through the Japan Marrow Donor Program [27]. On day 0, BM graft was infused without T-cell depletion; ABO major-mismatched or bidirectionally mismatched graft was processed to isolate mononuclear cell suspension using COBE Spectra (Gambro BCT, Lakewood, CO, USA) or CS-3000 Plus (Baxter Corp., Deerfield, IL, USA) according to the manufacture's instruction, while ABO minor-mismatched graft was plasma depleted before infusion. Eleven patients who were suspected to develop bacterial infection during the first week after transplantation or who were considered to be at high risk for infectious complications because of prior history of allogeneic transplantation received infusional or subcutaneous granulocyte colony-stimulating factor 5 μg/kg/day from day 7 until ANC exceeded 0.5 × 10^9/L.

Continuous intravenous administration of tacrolimus in a dose of 0.02 mg/kg/day was started on day −3 in patients receiving busulfan-based conditioning or on day −1 in patients receiving melphalan-based conditioning with therapeutic monitoring which targeted blood levels of 10–15 ng/ml at least until day 28 after transplantation, converted to twice-daily oral administration at an appropriate time to maintain trough levels between 5 and 10 ng/ml until day 100, followed by stepwise tapering over 3–6 months if active GVHD was absent. MTX at a dose of 5 mg/m^2 was intravenously injected on days 1, 3, and 6; MMF 30 mg/kg/day was orally administered in three divided doses from days 7 to 27. After day 28, MMF was discontinued without tapering if acute GVHD was absent or gradually tapered if ongoing acute GVHD was present. Patients who developed grade II–IV acute GVHD were initially treated with methylprednisolone or prednisolone at a dose of 1–2 mg/kg/day. All patients received supportive care including blood product transfusion and prophylaxis against opportunistic infections according to our institutional protocols [29].

2.5 Statistical analysis

Probabilities of neutrophil recovery, platelet recovery, and grade II–IV or grade III–IV acute GVHD were calculated by cumulative incidence estimates, treating death without the respective event as a competing risk [30]. Overall survival from the date of transplantation until the date of death from any cause was estimated by the Kaplan–Meier method; progression-free survival was estimated from the date of transplantation until the date of disease progression, relapse, or death from any cause. Data on patients who were alive at the time of last follow-up were censored. All statistical analyses were performed using STATA version 10 software (Stata Corp., College Station, TX, USA) based on dataset available on 10 January 2008.

3 Results

3.1 Patient and transplant characteristics

Table 1 shows the characteristics of the patients and transplantation procedures. A total of 17 patients (86%) had an advanced disease at transplantation, while the remaining 4 patients had an early disease. With respect to the compatibility at *HLA-A*, *-B*, and *-DRB1*, five patients (24%) received a single-allele mismatched graft, three had a mismatch at *HLA-A* and two at *HLA-DRB1*. Two of these five patients were found to have an additional allele mismatch at *HLA-C*. The median total number of nucleated cells included in the collected BM graft was 3.0 (range, 1.2–4.0) × 10^8 per kg of the recipient's body weight.

3.2 Engraftment

All patients achieved successful donor cell engraftment. The cumulative incidence of neutrophil recovery >0.5 × 10^9/L by day 35 was 100%, with a median time of 19 days (range, 13–35 days) (Fig. 1a). The cumulative probability of platelet recovery >20 × 10^9/L by day 42 was 81%, with a median time of 26 days (range, 13–91 days) (Fig. 1b). Two patients had experienced relapse on days 39 and 67 after transplantation without platelet recovery. No secondary graft failure was observed.

3.3 Acute and chronic GVHD

Acute GVHD was evaluable in all the patients. A total of seven patients developed grade II–IV acute GVHD: grade II in 6 and grade IV in 1. Cumulative incidence of developing grade II–IV acute GVHD at day 100 after transplantation was 33% (95% CI, 15–53%), and that of grade III–IV acute GVHD was 5% (95% CI, 0.3–20%)

Table 1 Patient and transplant characteristics

	$n = 21$
Median recipient age (range) (years)	52 (24–66)
Recipient sex, *n*	
Female/male	12/9
Diagnosis, *n*	
Acute myeloid leukemia	5
Myelodysplastic syndrome	4
Adult T-cell leukemia/lymphoma	4
Follicular lymphoma	3
Hodgkin lymphoma	1
Plasma cell myeloma	2
Chronic active EBV infection	1
Extranodal NK/T-cell lymphoma	1
Disease status at transplantation, *n*	
Early disease	
CR1	1
Untreated	3
Advanced disease	
CR > 1	4
PR	8
Progressive disease	5
Median donor age (range) (years)	34 (20–48)
HLA matching (at HLA-A, -B, -DRB1), *n*	
Match	16
Single-allele mismatch	5
ABO incompatibility, *n*	
Match	9
Minor	3
Major	5
Bidirectional	4
Conditioning, *n*	
Fludarabine + busulfan + 4 Gy TBI	14
Fludarabine + busulfan	1
Fludarabine + melphalan + 2 Gy TBI	2
Fludarabine + melphalan	4

EBV Epstein-Barr virus, *CR* complete remission, *PR* partial remission, *TBI* total-body irradiation

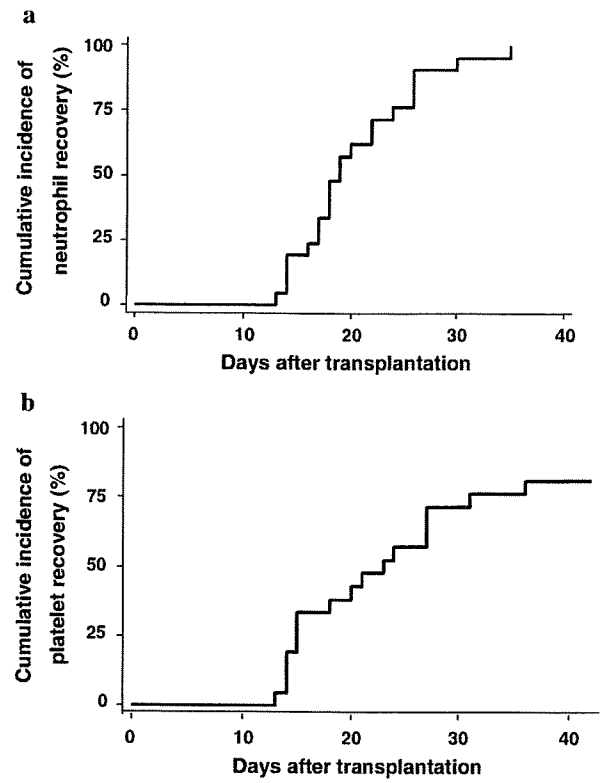

Fig. 1 Cumulative incidence of neutrophil recovery (**a**) and platelet recovery (**b**)

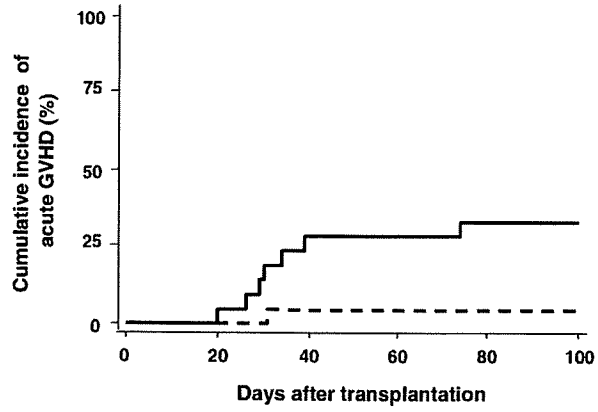

Fig. 2 Cumulative incidences of grade II–IV (*solid line*) and grade III–IV (*dashed line*) acute GVHD

(Figs. 2, 3). Chronic GVHD was observed in 11 of 20 (55%) evaluable patients who survived 100 days after transplantation: limited type in 6 and extensive type in 5.

3.4 Transplant-related toxicities and infectious complications

Transplant-related organ toxicities during the first 100 days after transplantation are shown in Table 2. Mild to moderate gastrointestinal symptoms considered to be associated with preparative regimens were frequently observed, although other adverse events were mostly moderate. One patient was required to discontinue MMF on day 9 because of grade III diarrhea.

Eighteen patients (86%) experienced 38 episodes of documented or suspected infectious complications (Table 3). Fourteen episodes of culture-negative neutropenic fever were reported. Five episodes of microbiologically documented bacterial infection were observed in four patients:

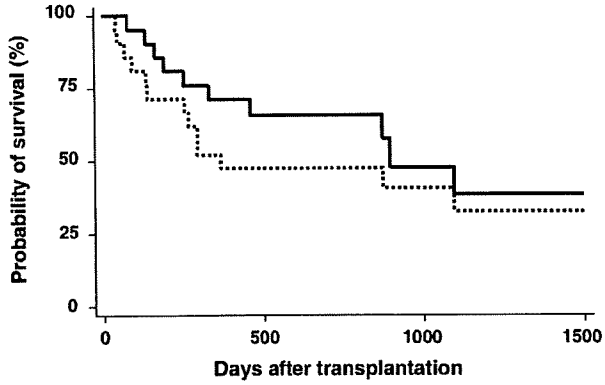

Fig. 3 Probabilities of overall survival (*solid line*) and progression-free survival (*dotted line*) after transplantation

Table 2 Transplant-related toxicities

	CTCAE grade I–II	CTCAE grade III
Vomiting	3 (14%)	6 (28%)
Stomatitis	5 (24%)	6 (28%)
Diarrhea	9 (43%)	3 (14%)
Liver dysfunction	5 (24%)	3 (14%)
Renal dysfunction	3 (14%)	0 (0%)
Headache	0 (0%)	2 (10%)
Pleural effusion	2 (10%)	0 (0%)
Myalgia	2 (10%)	0 (0%)

CTCAE common terminology criteria for adverse events

Table 3 Infectious complications

	No. of episodes
Culture-negative febrile neutropenia	14
Bacteremia	4
Clostridium difficile-associated diarrhea	1
Phlegmone	1
Neutropenic enterocolitis	1
CMV antigenemia	11
Cystitis	5
Aseptic meningitis	1
Total	38

CMV cytomegalovirus

bloodstream infection ($n = 4$) and *Clostridium difficile*-associated enteritis ($n = 1$). Two episodes of suspected bacterial infections were reported: phlegmone ($n = 1$) and neutropenic enterocolitis ($n = 1$). Eleven patients became positive for cytomegalovirus (CMV) antigenemia; one of them developed CMV-associated hepatitis and enteritis. Adenovirus was detected from the urine from one of five patients who developed cystitis. One patient experienced aseptic meningitis. No varicella-zoster virus infection was observed. There was no death directly attributable to infectious events until day 100.

Six patients developed non-infectious pulmonary complications between 3 and 14 months after transplantation: bronchiolitis obliterans ($n = 2$), bronchiolitis obliterans-organizing pneumonia ($n = 1$), diffuse alveolar hemorrhage ($n = 1$), and idiopathic interstitial pneumonia ($n = 2$). One patient developed secondary gastric cancer at 34 months after transplantation.

3.5 Survival and treatment-related mortality

Eleven patients were alive and 10 of them were disease-free at a median follow-up of 24 months (range, 3–31 months). Among four patients who had an early disease at transplant, one experienced relapse on day 292. Fifteen of 17 patients who had an advanced disease maintained or attained remission after transplantation, but seven of them eventually relapsed between days 67 and 363. Two of the relapsed patients received donor lymphocyte infusion after chemotherapy, and durable remission lasting more than 16 months was observed in one patient.

Ten patients were deceased between 76 and 1093 days after transplantation. Six patients succumbed to disease progression and four patients died of treatment-related complications including interstitial pneumonia ($n = 1$), bronchiolitis obliterans followed by diffuse alveolar damage ($n = 1$), intracranial hemorrhage during exacerbation of bronchiolitis obliterans ($n = 1$), and secondary gastric cancer ($n = 1$). The probabilities of overall survival and progression-free survival at 3 years after transplantation were 38% (95% CI, 14–63%) and 33% (95% CI, 12–55%), respectively.

4 Discussion

In this study, we evaluated the efficacy of a combination of tacrolimus, minidose MTX, and MMF as post-transplant immunosuppression in RIC transplantations using BM grafts from an HLA-A, -B, -DR antigen compatible unrelated donor. This triple regimen conferred stable donor cell engraftment, low risk of severe acute GVHD, and encouraging overall survival with acceptable toxicity profiles.

Recent introduction of RIC has provided the opportunity to enjoy long-term disease-free survival in patients with hematologic malignancies who were previously ineligible for allogeneic HSCT because of elder age or pre-existing comorbidity. It has been shown that RIC HSCT using alternative stem cell source is a feasible treatment option when an HLA-matched related donor is not available, albeit at the expense of substantial risk of more serious

transplant-related complications. Among the first 285 patients who underwent unrelated RIC HSCT through the National Marrow Donor Program, the respective incidence rates of primary graft failure, grade III–IV acute GVHD, and treatment-related mortality at 3 months after transplantation were 11, 22, and 19%, respectively [7].

It should also be noticed that RIC transplantations using BM as a stem cell source have been reported to be associated with a higher risk of graft failure when compared with those using cytokine-mobilized PBSC, especially in the unrelated donor setting [6, 7]. As compared with BM, PBSC grafts usually contain more than ten times higher number of T-cells and 2–4 times greater number of $CD34^+$ cells, which would have a beneficial impact on successful engraftment after RIC [31]. In a study which compared the engraftment kinetics after transplantation of PBSC and BM with an identical non-myeloablative conditioning, the number of patients who achieved full donor chimerism was significantly lower in the BM group [32]. These observations suggested that, to improve the outcomes after RIC transplantation using BM grafts from unrelated donors, it is important to develop more optimal post-transplant immunosuppressive protocol which can effectively prevent graft rejection as well as severe GVHD.

In preclinical canine models and clinical experiences of HSCT after truly non-myeloablative regimen using low-dose TBI with or without fludarabine as pre-transplant conditioning, post-transplant administration of MMF was shown to improve the rate of successful donor cell engraftment [11, 33]. Therefore, we hypothesized that the addition of MMF to the standard immunosuppression with tacrolimus plus minidose MTX might facilitate engraftment after unrelated BM allografting with RIC. In support of this hypothesis, all the patients in this study achieved durable donor cell engraftment without experiencing serious morbidity associated with delayed hematopoietic recovery or late graft failure. However, this promising result awaits further validation because the probability of engraftment can also be influenced by the type and intensity of RIC regimens or by the use of pre-transplant anti-thymocyte globulins or T-cell-depleting monoclonal antibodies. Onishi et al. reported the outcomes of unrelated BM transplantation after RIC with fludarabine, busulfan, and 4 Gy TBI among a cohort of 17 patients with various hematologic malignant diseases. Although all the patients in their report initially achieved successful engraftment with the use of conventional post-transplant immunosuppression composed of cyclosporine and MTX, 2 of them subsequently developed secondary graft failure [8]. This observation suggests that the intensification of conditioning with 4 Gy TBI does not always confer sustained engraftment, at least in the setting of unrelated marrow transplantation.

In contrast, the role of MMF in ameliorating acute GVHD has been controversial at least when administered solely with calcineurin inhibitors. Recently, Koh et al. [34] reported that the post-grafting MTX combined with cyclosporine and MMF significantly reduced the risk of grade III–IV acute GVHD as compared with a combination of cyclosporine and MMF. Consistent with their experience, the cumulative incidence of severe acute GVHD after our triple combination was 5%, encouragingly lower than those previously reported in the analysis of unrelated BMT through the Japan Marrow Donor Program, while the incidence of extensive chronic GVHD was apparently similar [27, 35]. However, an important concern regarding the intensification of post-transplant immunosuppressive regimen is an increased risk of infection or relapse. In this study, a substantial proportion of patients developed manageable infections and experienced disease progression within 1 year after transplantation. Although the incidence rates of these events might be adversely affected by the high proportion of patients who had an advanced disease at transplantation, further studies are needed to elucidate whether our triple immunosuppressive regimen may increase the risk of infectious complications or may compromise the graft-versus-tumor effect after RIC HSCT [36, 37].

An unresolved issue in the present study is a pharmacokinetic/pharmacodynamic profile of MMF when combined with tacrolimus and MTX. The increased mean total plasma MPA concentrations at steady state were reported to be associated with higher donor cell chimerism after unrelated non-myeloablative transplantation, while the lower MPA levels were shown to be a predictor of graft rejection [38]. We administered MMF in three divided doses rather than in twice-daily doses because the former is more likely to confer higher mean total MPA concentrations [38, 39]. Because it is speculated that the bioavailability of oral MMF is highly variable depending on the degree of gastrointestinal mucosal damage and donor-recipient pharmacogenomic backgrounds [40], it is important in the future studies to evaluate the association of MPA pharmacodynamics with the risk of post-transplant immunologic complications such as graft rejection, acute GVHD, and infections. Furthermore, appropriate dosing of MMF would be affected by the type of combined calcineurin inhibitor: cyclosporine is reported to decrease MPA exposure due to delay of the excretion of the MPA metabolites, while tacrolimus is less likely to cause drug interaction with MPA [41, 42].

In conclusion, our study demonstrated the feasibility and efficacy of using a triple combination of tacrolimus, minidose MTX and MMF as post-grafting immunosuppression after RIC BM transplantation from unrelated donors. Because this triple regimen conferred high

probability of sustained donor engraftment with an acceptable risk of transplant-related complications, further studies are warranted to confirm its efficacy in a larger population including patients who receive HLA-mismatched family donor grafts or unrelated cord blood units with dose-reduced conditioning.

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ORIGINAL ARTICLE

GATA-1 and GATA-2 binding to 3′ enhancer of *WT1* gene is essential for its transcription in acute leukemia and solid tumor cell lines

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Although oncogenic functions and the clinical significance of Wilms tumor 1 (*WT1*) have been extensively studied in acute leukemia, the regulatory mechanism of its transcription still remains to be determined. We found a significant correlation among the amounts of *WT1*, *GATA-1* and *GATA-2* mRNAs from leukemia and solid tumor cell lines. Overexpression and small interfering RNA (siRNA) transfection experiments of *GATA-1* and *GATA-2* showed that these *GATA* transcription factors could induce WT1 expression. Promoter analysis showed that the 5′ promoter did not explain the different *WT1* mRNA levels between cell lines. The 3′ enhancer, especially the distal sites out of six putative GATA binding sites located within the region, but not the intron 3 enhancer, were essential for the *WT1* mRNA level. Electrophoretic mobility shift assay (EMSA) showed both GATA-1 and GATA-2 bound to these GATA sites. Besides acute leukemia cell lines, solid tumor cell lines including, TYK-nu-cPr also showed a high level of *WT1* mRNA. We showed that *GATA-2* expression is a determinant of *WT1* mRNA expression in both TYK-nu-cPr cells and HL60 cells without GATA-1 expression. Taken together, these results suggest that GATA-1 and/or GATA-2 binding to a GATA site of the 3′ enhancer of *WT1* played an important role in *WT1* gene expression.
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Introduction

Wilms tumor 1 (*WT1*) gene was isolated as a tumor suppressor gene responsible for Wilm's tumor, a kidney neoplasm of childhood. The gene product, WT1, represses the transcription of growth factors, growth factor receptors and other genes.[1–3] On the other hand, *WT1* has been regarded as an oncogene for leukemia and some solid tumors because high expression levels of *WT1* mRNA were observed in these diseases, and *in vivo* and *in vitro* studies showed the positive roles it plays in cell growth and transformation.[4–8] *WT1* mRNA is reportedly a good clinical marker for disease progression, diagnosis and detection of the minimal residual diseases of myelodysplastic syndromes and leukemia.[9–11] Thus, depending on the cells and situations expressing WT1, it displays both anti-oncogenic and oncogenic characters.[12] Despite the fact that the oncogenic functions of WT1 have been well documented, its gene-expression mechanism remains undetermined.

In the promoter analysis of *WT1* gene, Cohen *et al.*[13] reported the importance of Sp1 for mRNA expression, although the promoter region may not determine the tissue-specific manner of *WT1* expression.[13,14] Regarding this point, the 3′ enhancer located >50 kb downstream of the promoter was identified as increasing the basal transcription rate of the *WT1* promoter in the erythroleukemia cell line K562.[14] Subsequently, Wu *et al.*[15] showed that GATA-1 may bind to this enhancer, which is reported to be specific in hematopoietic cells.[16] To date, in addition to Sp1 and GATA-1, PAX2 and PAX8 are also reported to be the transcription factors that regulate *WT1* gene expression.[17–20] Dehbi *et al.*[17] reported the binding sites of PAX2 and PAX8 in the 5′ promoter of *WT1* gene. Zhang *et al.*[20] indicated the importance of the intron 3 enhancer of *WT1* gene, which was activated by GATA-1 and Myb.

These earlier results suggest that between the cells analyzed, the regulation of *WT1* gene expression might be complex and heterogeneous. Furthermore, it was not clear what the major determinant of *WT1* mRNA level is either in leukemia or in solid tumors. Although the GATA family has been shown to play critical roles in erythroid and other hematopoietic lineages,[21,22] GATA-1 expression is low in M3, M4 and M5 of acute myelogeneous leukemia,[23] in which WT1 expression has been observed. Therefore, GATA-1 might not be sufficient to maintain full *WT1* gene expression. For another transcription factor candidate, we focused on GATA-2, which is presented in cells at the early-differentiated stage of hematopoietic lineages. We examined the relationship between *WT1*, *GATA-1* and *GATA-2* mRNA from 20 leukemia cell lines and 26 acute leukemia bone marrows, as well as purified stem cell and progenitor cell fractions from two normal bone marrows. We also examined solid tumor cell lines with high *WT1* expressions. There are no reports describing the regulatory mechanism of *WT1* gene expression in these cell lines.

On the basis of our results, we discussed the functional relationship between Sp1 and *WT1* expression and that between GATA and *WT1* expression in leukemia cell lines and several solid tumor cell lines.

Materials and methods

Clinical samples and cell lines

After obtaining informed consent, bone marrow cells were collected from 23 acute myeloid leukemia (AML) patients and three ALL patients without Ph[1] chromosome. Normal control

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samples were derived from malignant lymphoma patients without bone marrow involvement as described earlier.[24,25] Our analysis of *WT1, GATA-1* and *GATA-2* was approved by IRB (approved number 99) of the Nagoya University School of Medicine. Some leukemia cell lines were provided by Dr Hirokazu Nagai (National Hospital Organization, Nagoya Medical Center, Nagoya, Japan). Solid tumor cell lines with high *WT1* mRNA levels[8] were the generous gifts of Dr Haruo Sugiyama (Osaka University Graduate School of Medicine, Osaka, Japan).

Quantitative reverse transcriptase-PCR

Quantitative reverse transcriptase-PCR was performed as described.[24,25] *WT1* mRNA was measured using the Light Cycler system (Roche Diagnostics, Mannheim, Germany). Each primer set and PCR conditions have been described before.[24,25] *GATA-1, GATA-2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs were measured with a Power SYBR Green master mix using the ABI PRISM 7000 sequence detection systems (Applied Biosystems Japan Ltd, Tokyo, Japan). After producing first-strand cDNA, quantitative PCR was carried out using the following primer sets: *GATA-1*, forward 5'-CCCAAGAAGCGCCT GATTGT-3'; reverse 5'-GTGTAGCTTGTAGTAGAGGCCGC-3'. *GATA-2*, forward 5'-CGTTCCTGTTCAGAAGGC-3'; reverse 5'-G TTCTGCCCATTCATCTTGT-3'. *GAPDH*, forward 5'-CAGGAGC GAGATCCCTCCAA-3'; reverse 5'-CCCCCTGCAAATGAGCCC-3'. PCR conditions were GATA-1, 95 °C for 10 s followed by 56 °C for 15 s; GATA-2, 95 °C for 10 s followed by 56 °C for 15 s; and GAPDH, 95 °C for 10 s followed by 63 °C for 15 s. Forty cycles were used for *WT1, GATA-1, GATA-2* and *GAPDH*. *GAPDH* was used as the internal control for both clinical samples and cell lines. Relative mRNA was calculated as the respective mRNA/*GAPDH* mRNA in the log scale.

Western blotting

Western blotting of WT1, GATA-1, GATA-2 and Sp1 was carried out using the anti-WT1 antibody (clone 6F-H2, Dakocytomation, CA, USA), anti-GATA-1 (sc-265), anti-GATA-2 (sc-9008) and anti-Sp1 (sc-59) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), respectively. We used the ECL chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) for signal detection.

Rapid amplification of 5' cDNA ends (5' RACE)

The transcription initiation point of *WT1* was determined as described in the Supplementary information.

Cloning of WT1 promoter and enhancer regions

We obtained an ~1.8-kb fragment covering the 5' region of exon1 of *WT1* using the 5' rapid amplification of cloned ends. This fragment was inserted into the *Kpn*-I and *Bgl*-II sites of pGL3 basic vector (Promega, Madison, WI, USA), and used for the promoter analysis (−1807/Luc). Truncation and mutation of *WT1* promoter were prepared by the PCR-based method. Similarly, the intron 3 and 3' enhancers of the *WT1* were also obtained by the PCR-based method. Production of the truncation and mutation of 5' promoter and enhancer is described in the Supplementary information.

Promoter and enhancer analysis

K562 (1×10^6), Jurkat (1×10^5), TYK-nu-cPr (1×10^5) and HepG2 (1×10^5) cells were transfected with 1 μg of reporter plasmid containing various lengths of the 5' promoter and/or enhancer region of *WT1* gene and 1 μg of β-galactosidase expression vector (Promega). The lipofectin reagent (Invitrogen, Carlsbad, CA, USA) was used for K562. The calcium precipitation method was used for TYK-nu-cPr and HepG2. Trans IT-Jurkat (Mirus Bio Co., Madison, WI, USA) was used for Jurkat cells. After 48 h, cell lysates were prepared. Luciferase activity was normalized with the β-galactosidase activity.

DNA transfection

Transfection of the expression vector was carried out using Trans IT-Jurkat, for Jurkat, and the calcium precipitation method for TYK-nu-cPr cells. *GATA-1* and *GATA-2* expression vectors were obtained from Dr Ritsuko Shimizu (Tsukuba University, Tochigi, Japan) and Dr Haruhiko Asano (Chubu University, Kasugai, Aichi, Japan), respectively. *GATA-1* cDNA or *GATA-2* cDNA was transferred into pcDNA3.1 vector (Invitrogen).

RNA interference

K562 cells (5×10^5) were transfected with small interfering RNA (siRNA) (final 100 nM) using Oligofectamine reagent (Invitrogen). GATA-1 siRNA (ID no. 3197) and GATA-2 siRNA (ID no. 145419) were purchased from Ambion (Austin, TX, USA). Scrambled siRNA was purchased from Dharmacon (GE Healthcare Sciences, Tokyo, Japan).

Electrophoresis mobility shift assay

Nuclear extract was prepared from K562, Jurkat, Daudi, HL-60, TYK-nu-cPr, Sw480, Az521 and HepG2 cells. Electrophoretic mobility shift assay (EMSA) was performed as described earlier.[26] For the supershift experiment, anti-GATA-1 or anti-GATA-2 antibody (Santa Cruz Inc.) was added to the nuclear extract for 30 min at room temperature before mixing with biotin-labeled probes as described below. Forward, 5'-CATTTA **TATC**AGCCGTTTT**TATC**TTTTCCTG-3'; reverse, 5'-CAGGAAA A**GATA**AAAACGGCT**GATA**TAAATG-3' (Bold letters are the GATA binding motifs). In some experiments, mutated oligo was used. Proximal-mutated GATA: forward, 5'-CATTT**CCAAA** AGCCGTTCCAAATTTTCCTG-3'; reverse, 5'-CAGGAAAAGT TTGGAACGGCT**TTTGG**AAATG-3'. Distal mutated GATA: forward, 5'-CATTTATATCAGCCGTT**CCAAA**TTTTCCTG-3'; reverse, 5'-CAG GAAAAG**TTTGG**AACGGCTGATATAAATG-3' (Bold letters are the mutated GATA binding sites). Biotin label was attached to the 3' end of each forward probe (Sigma Genosys, Hokkaido, Japan).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was carried out as described earlier.[26] K562, Jurkat and TYK-nu-cPr cells were used for the crosslinking with formaldehyde. For the immunoprecipitation, normal mouse IgG, anti-GATA-1-antibody (Santa Cruz, final concentration; 3 μg/μl) or anti-GATA-2-antibody (Santa Cruz) was added and incubated at 4 °C overnight. Immunocomplexes were extracted and crosslinking was reversed by heating the elutes at 65 °C overnight. The eluates were then digested with proteinase K at 50 °C for 5 h and extracted with phenol/chloroform/isoamyl alcohol. DNA was purified by ethanol precipitation. The WT1 enhancer region was amplified by PCR using primers 5'-GGGAATTCGACTCATTTATATCAG CCGTTTT-3' (forward) and 5'-GGGTCGACCCTGGCTCTTTCC GACTC-3' (reverse).

Results and discussion

Correlation between mRNA levels of WT1 *and* GATA-1/GATA-2 *in acute leukemia clinical samples and cell lines*

In Figure 1, we analyzed 20 established leukemia cell lines to determine the correlation between *WT1* and *GATA-1/GATA-2* (Figures 1a and c). Seventy-five percent of the cell lines showed high mRNA expression (more than 10^{-3} of *GAPDH* mRNA), whereas others (such as Daudi and U937) showed a very low or undetectable level of *WT1* mRNA. *GATA-1* mRNA was observed in 10 out of the 20 cell lines analyzed, with the expression levels being heterogeneous. *GATA-2* mRNA was also observed in 13 out of those 20 cell lines. The correlation index between *WT1* and *GATA-1* or *WT1* and *GATA-2* was significant, suggesting a positive relationship between *WT1* and *GATA-1/-2* mRNA (Figure 1 lower part). The correlation between *GATA-1* and *GATA-2* mRNA was also positive. When we examined solid tumor cell lines (SW480, Az521 and TYK-nu-cPr), which express high *WT1* mRNA[8] (Figure 1b), we found that those cell lines with high *WT1* mRNA showed high *GATA-2*, but not *GATA-1* mRNA. We also found a high

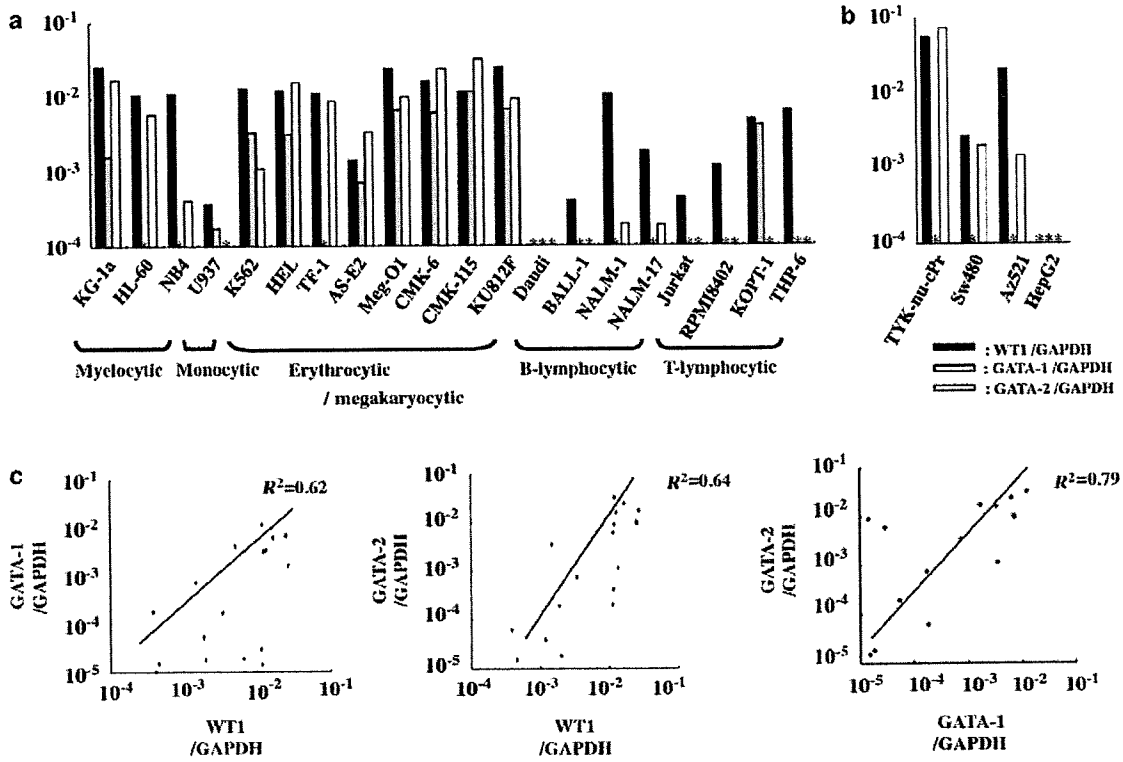

Figure 1 *WT1, GATA-1* and *GATA-2* mRNA in leukemia cell lines (a) and solid tumor cell lines (b). Relative *WT1, GATA-1* and *GATA-2* mRNA levels were examined by a quantitative reverse transcriptase-PCR. The internal control was *GAPDH*. The asterisks denote below the detection limit. The lower panels (c) show the correlation between *WT1* and *GATA-1, WT1* and *GATA-2*, and *GATA-1* and *GATA-2*, respectively.

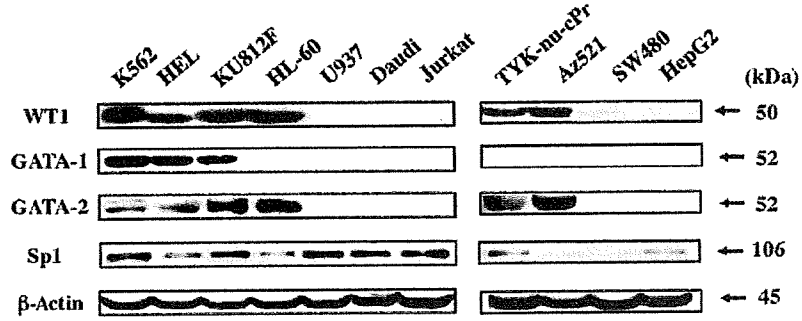

Figure 2 WT1, GATA-1, GATA-2 and Sp1 protein levels of representative cell lines. Left: Representative leukemia cell lines with high (K562, HEL, KU812F and HL60 cells) and low *WT1* mRNAs (U937, Daudi and Jurkat cells) were examined for their WT1, GATA-1, GATA-2 and Sp1 protein expressions by western blotting as described in Materials and methods. β-Actin was shown as the internal control. Right: solid tumor cell lines with high *WT1* mRNA (TYK-nu-cPr, Az521, SW480) as well as the negative control of HepG2 cells were analyzed for their WT1, GATA-1 and GATA-2 protein levels.

expression of *GATA-3* in Jurkat cells (data not shown), but its expression levels in the cell line panels were not examined further, as it was reported to express mainly in T lymphocytes and the embryonic brain.[27,28] We did not analyze *PAX* family genes because there are clear examples of tumor cell lines in which *PAX8* is not expressed but *WT1* is, or vice versa.[29,30]

Supplementary Figure 1 illustrates the relative mRNA levels of *WT1, GATA-1* and *GATA-2* from 23 AML and 3 ALL patient bone marrows. Most leukemia patients (85%) showed *WT1* mRNA levels of more than 10^{-2} of *GAPDH* mRNA, whereas the absence of *WT1* mRNA (below our detection limit) was observed only in one AML patient. *GATA-1* mRNA was very low in one AML case, whereas *GATA-2* mRNA was well observed in most. A low correlation index between *WT1* mRNA and *GATA-1/2* mRNA was obtained (data not shown) probably because of the heterogeneous percentage of blast cells within samples, and because of the remaining erythroid and megakaryocytic lineage cells.

We also examined *WT1, GATA-1* and *GATA-2* mRNA levels of both normal human hematopoietic stem cell fractions ($CD34^+$, $CD38^-$, Lin^-) and progenitor cell fractions ($CD34^+$, $CD38^+$, Lin^-) purified from two independent samples according to the method described in the Supplementary information. *WT1* mRNA levels of normal stem cell and progenitor cell fractions were significantly lower than those of AML samples with high *WT1* mRNA, whereas the *GATA-1* and *GATA-2* mRNA levels were almost equivalent to those of AML samples. In normal stem cell/progenitor cells, factors other than GATA-1/2 might also be relevant to their *WT1* mRNA expression. The contribution of GATA-1/2 to *WT1* mRNA expression of normal stem cell/progenitor cell fraction should be further analyzed in future experiments.

As to the *WT1* mRNA of the stem cell fraction, Hosen *et al.*[31] described the very low frequency of normal CD34-positive-hematopoietic progenitor cells with *WT1* mRNA level similar to those in leukemia cells. Our current study supports their results. Mouthon *et al.*[32] reported their *in situ* hybridization, suggesting the gradual increase of *GATA-1* mRNA from stem cells to immature erythroblasts and megakaryocytes. In our results, increase of *GATA-1* mRNA was observed from stem cell fraction to progenitor cell fraction. Furthermore, Maratheftis *et al.*[33] reported the upregulation of *GATA-1* mRNA of $CD34^+$ cells in myelodysplastic syndrome compared with that of normal control.

For precise and further analysis of a pure leukemia population, we used leukemia cell lines. Figure 2 illustrates the WT1, GATA-1, GATA-2 and Sp1 protein levels of representative cell lines with either high or low *WT1* mRNA. In these cell lines, WT1 protein levels were well correlated with *WT1* mRNAs. In WT1-high-leukemia cell lines, GATA-1 and GATA-2 protein levels were higher than those in WT1-low-leukemia cell lines. In the case of SW480, factors other than GATA1 and GATA2 might be involved in its WT1 expression.

5'-promoter analysis of WT1 *gene*

We determined the *WT1* transcription start site of K562 cells using the 5' rapid amplification of cloned ends method.[26] The

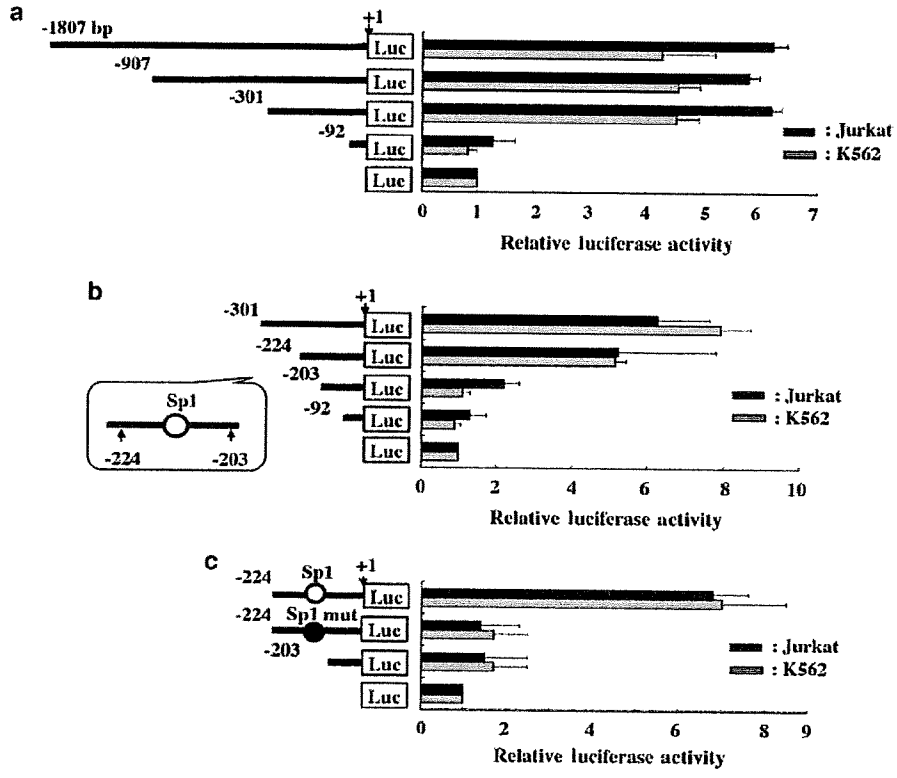

Figure 3 Promoter analysis of the 5' region of *WT1* gene. Using either K562 or Jurkat cells, the promoter activity of *WT1* gene was examined by transfecting the pGL3 basic reporter vector containing various lengths of the 5' promoter region as described in Materials and methods (**a**). Results were shown as the ratio of luciferase activity/β-gal. Data of pGL3 basic without 5' promoter were regarded as 1.0. The Sp1 site located between −224 and −203 bp is illustrated in (**b**). The solid circle in (**c**) shows mutated Sp1 site. The mean ± s.d. was calculated from three independent experiments.

start site of K562 cells was 185 bp downstream of the reported site in the National Center for Biotechnology Information database and 380 bp downstream of the one reported earlier.[13,14] On the basis of our result, we cloned the −1.8-kb region of *WT1* 5′ promoter. Figure 3 showed that the most potent promoter activity was located between −224 and −203 bp from exon 1 (Figures 3a and b). The introduction of a mutation into a putative Sp1 site in this region (Figure 3c) clearly showed this Sp1 site is essential for the 5′-promoter activity of *WT1*, which is consistent with the result of Cohen *et al.*[13] It also suggests that this promoter activity was not the sole determinant of the *WT1* expression, because K562 and Jurkat cells, which were associated with the high and low *WT1* expression, respectively (Figure 2), showed similar promoter activity, and the Sp1 protein level did not correlate with the WT1 protein level (Figure 2).

Enhancer analysis of WT1 *gene*

On the basis of results in Figures 2 and 3 as well as earlier reports suggesting the importance of intron 3 and 3′ enhancers,[15,20] we prepared luciferase reporter vectors containing −303 bp of the 5′-promoter region combined with the intron 3 and/or putative 3′ enhancer illustrated in Figure 4a. It is clear that the 3′ enhancer is potent in K562 cells with high *WT1* mRNA, but not in Jurkat or HepG2 cells with low *WT1* mRNA (Figure 4b). Interestingly, this 3′ enhancer was also active in TYK-nu-cPr cells, a solid tumor cell line with high *WT1* mRNA. In our analysis, the intron 3 enhancer did not play a major role in *WT1* gene expression, as was described earlier,[20] though some additive effect was detected in the presence of 3′ enhancer. We further analyzed 3′ enhancer by preparing various truncated forms of this region. Figure 4b showed that the distal region containing three out of six GATA sites was important. In earlier studies, Fraizer *et al.*[14] reported the importance of 3′ enhancer, and Wu *et al.*[15] confirmed their results using EMSA. However, the minimal responsive GATA site within this region has not been determined. Subsequent experiments using a mutated GATA motif introduced into these putative sites (Figure 4c) clearly showed that the minimal region in determining the total promoter/enhancer activity was the most distal GATA site of this enhancer.

The effects of transient expressions of *GATA-1* and *GATA-2* on *WT1* mRNA and *WT1* promoter and on WT1 protein of Jurkat and HepG2 cells are illustrated in Figure 5. *GATA-1* overexpression induced *WT1* mRNA in both Jurkat cells (1.9 times compared with mock-transfected cells) and HepG2 cells (2.0 times), whereas *GATA-2* overexpression increased WT1 mRNA in both Jurkat cells (1.7 times compared with mock-transfected cells) and HepG2 cells (2.4 times), respectively. *GATA*-induced WT1 protein expression was clearly observed in HepG2 cells (Figure 5b). The increase of WT1 protein in Jurkat

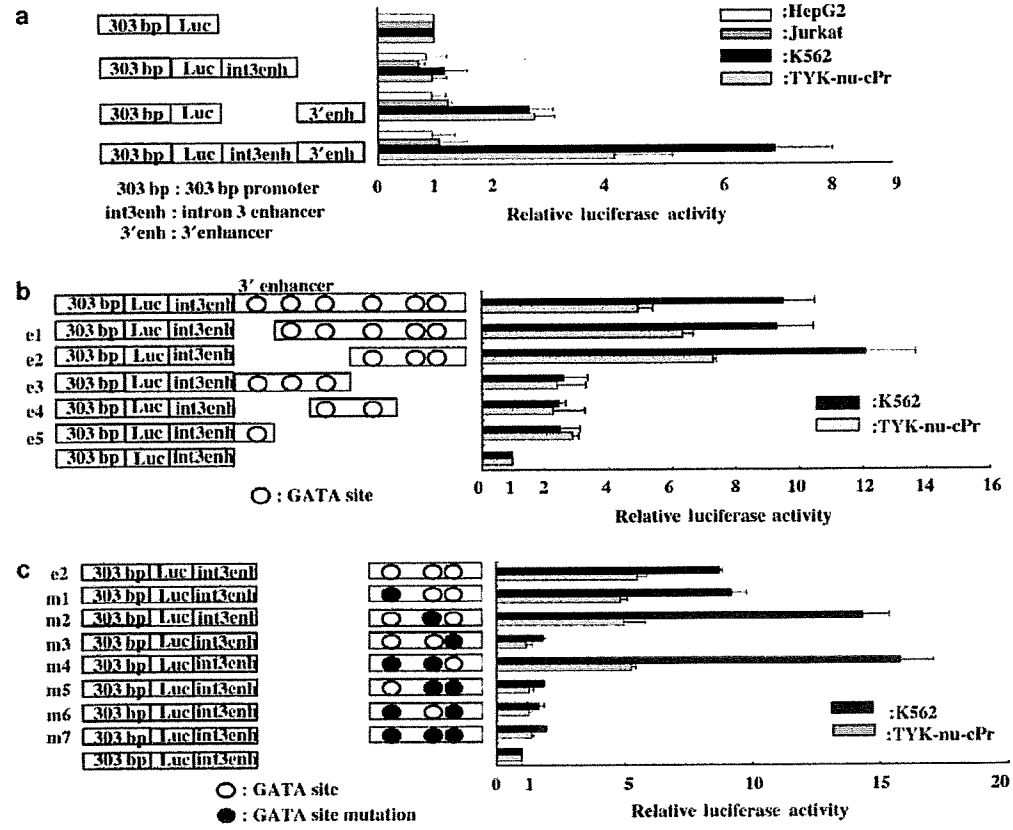

Figure 4 Effects of the intron 3 and 3′ enhancers of *WT1* gene. Effects of the intron 3 and 3′ enhancers were analyzed by luciferase reporter assay using various combinations of the promoter and enhancers as shown in (**a**) left. The sequence information was derived from earlier publications[14,15,20] and is described in Supplementary information. The mean ± s.d. was calculated from three independent experiments. Data of the relative luciferase activity of control vector (without enhancer elements) of each cell line were regarded as 1.0. In (**b** and **c**) deletion and mutation were introduced as illustrated in the left part of each panel. The open circle denotes wild-type GATA site, whereas the solid circle shows mutated GATA site. Data of the relative luciferase activity from luc vector containing only 5′ promoter and intron 3 enhancer were regarded as 1.0.