medium from WEHI 3B cells (WEHI-CM) as a source of IL-3. *JAK2V617F*-Ba/F3 cells were maintained in the absence of IL-3. JAK Inhibitor I (Calbiochem) was used at a final concentration of 0.5 mM. Murine *STAT5 1*6* mutant (constitutively active) and wild type *STAT5* expression pMX retrovirus vectors [10] were provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan). pEFHA STAT3 [11] was provided by Dr. T. Hirano (Osaka University, Osaka, Japan). Constitutive active STAT3, DMsam STAT3C-IRES-EGFP [12], was provided by Dr. A. Iwama (Chiba University, Chiba, Japan). Wild-type and constitutively active *STAT3* cDNA were cut out from the original vector and inserted into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA), and used for DNA transfection. To establish stable transfectants of *STAT3* and *STAT5*, Fugene 6 (Roche Diagnostics, Basel, Swiss) was used for DNA transfection and transformants were selected by G418. Retroviral *BCR/ABL* expression vector [13] and its control vector were a generous gift from Prof. C. Eaves (Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada). *BCR/ABL*-Ba/F3 and MIG-mock-Ba/F3 were established by transfection followed sorting of GFP positive clones by flow cytometry.

Western blotting. Western blotting was performed as described previously [14]. The antibodies used were anti-JAK2 (M-126, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-STAT3 (S5933, SIGMA, St. Louis, MO, USA), anti-STAT5A (S6058, SIGMA), anti-p-STAT5A/B (S5058, SIGMA), anti-p27^{Kip1} (C-19, Santa Cruz), anti-p15 (4822, Cell Signaling, Beverly, MA, USA), anti-CDK4 (2906, Cell Signaling), anti-CDK6 (3136, Cell Signaling), anti-p21^{WAF1} (C-19, Santa Cruz), anti-Cyclin D1 (2926, Cell Signaling), anti-Cyclin D3 (2936, Cell Signaling), anti-β-actin (Cytoskelton Inc., Denver, CO, USA), anti-p-JAK2 (Cell Signaling), anti-p-STAT3 (Cell Signaling) and anti-SKP2 (Santa Cruz, H-435).

Semi-quantitative RT-PCR. Semi-quantitative reverse transcription (RT)-PCR was performed according to a method described previously [14]. Primer sets are described in the Supplementary materials. The mRNA levels of *p27^{Kip1}* and *Skp2* are expressed as the ratio of *p27^{Kip1}/β-actin* and *Skp2/β-actin*, respectively.

Rapid amplification of 5′-cDNA ends (5′-RACE). The transcription initiation sites of mouse *p27^{Kip1}* and *Skp2* of Ba/F3 cells were determined with the RNA ligase-mediated rapid amplification method of 5′-cDNA ends (5′-RACE) using a Gene Racer kit (Invitrogen). Primer set was described in the Supplementary materials.

Cloning of promoter regions. The luciferase vector containing *Skp2* 5′-promoter (pGL2–2275) was a generous gift of Prof. K. Nakayama (Kyushu University Graduate School of Medicine, Fukuoka, Japan). This fragment was inserted into the SmaI and BglII sites of the pGL3 basic vector. Truncation and mutation of the *Skp2* promoter were prepared by a PCR-based method. The upper primers for truncated luciferase vectors of −1247, −916, −307, −167, −116 and −101 bp 5′-promoter/luc were 5′-GGGGCTAGCAAGAAGTAACTG ATGCAAAGA-3′, 5′-GGGGCTAGCATTCTAGGACAGGCTGTGGATT-3′, 5′-GGGGCTAGCAGTCCCGGGCGACCGTG-3′, 5′-CCTCCTCCTCCTTCA ATCC-3′, 5′-AGGGTTGGTCCGAAATCAG-3′ and 5′-GGGGCTAGC TCA GAGTGGAAGAACCCAG-3′. The lower primer was the pGL primer 2 of the pGL3 basic vector. A single underline denotes an added NheI enzyme site. The fragment was ligated to the NheI and NcoI (−1247, −916, −307 and −101 bp 5′-promoter/luc) or SmaI and NcoI (−167 and −116 bp 5′-promoter/luc) sites of the pGL3 basic vector. To introduce mutated STAT binding sites (Fig.. 3B), the following primer sets were prepared, and PCR was performed using the −916 bp 5′-promoter/luc as a template.

Primers (A) and (B) (for distal STAT binding site), 5′-CAGG TTTTCCTGGGCCCGGCGCTCAC-3′ and 5′-GTGAGCGCCGGGCCCAG GAAAACCT-3′.

Primers (C) and (D) (for proximal STAT binding site), 5′-CCTCCA GATACCCACGGCTCCCTGCG-3′ and 5′-CGCAGGGAGCCGTGGGTATC TGGAGG-3′.

A double underline denotes the mutated STATS site. To introduce the mutated distal and proximal STAT sites, a two-step PCR was performed. To obtain a distal STAT site mutation, PCR amplification was performed using the two primer sets, (RV primer 3 of the pGL3 basic vector and A, and B and pGL primer 2) with 916 bp 5′-promoter/luc as the template. Each PCR product was purified, and the mixture of these two PCR products was used for the second PCR template with the primer set of RV primer 3 and pGL primer 2. Proximal STAT mutation was obtained using a similar method. For the first PCR, two primer sets, RV primer 3 and C, and D and pGL primer 2, were used. Then, for the second primer set, RV primer 3 and pGL primer 2, was used with the first PCR products as the template.

Promoter analysis. Ba/F3 cells (1×10^6) were transfected with 5 μg of reporter plasmid containing various lengths of the 5′-promoter and 2 μg of β-galactosidase expression vector (Promega) using Lipofectin reagent (Invitrogen). After 24 or 48 h, cell lysates were prepared. Promoter activity was normalized with the β-galactosidase activity and was expressed as Luc/β-gal.

Electrophoresis mobility shift assay of Skp2. EMSA was performed as described previously [15]. For the supershift experiment, anti-STAT5 or anti-STAT3 antibody was added to the nuclear extract for 15 min at room temperature before mixing with biotin-labeled probes as described below. Forward, 5′-GGGAGTTGTGGGTATCTGG A-3′; reverse: 5′-TCCAGATACCCACAACTCCC-3′ (STAT binding motifs were underlined). In some experiments, mutated oligo was used. *Skp2* mutated *STAT* forward, 5′-GGGAGCCGTGGGTATCTGGA-3′; reverse, 5′-TCCAGATACCCACGGCTCCC-3′ (the mutated STAT binding motifs were double underlined). A biotin label was attached to the 3′ end of each forward probe (Sigma Genosys, Hokkaido, Japan).

Chromatin immunoprecipitation (ChIP) assay of Skp2. ChIP assay was performed as described previously [15]. *JAK2V617F*-Ba/F3 cells were used for the crosslinking with formaldehyde. For the immunoprecipitation, normal mouse IgG, anti-STAT3-antibody (final concentration: 3 μg/μl) or anti-STAT5-antibody (final concentration: 3 μg/μl) was added and incubated at 4 °C overnight. Immunocomplexes were extracted, and crosslinking was reversed by heating elutes at 65 °C overnight. 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 promoter region was amplified by PCR using primers 5′-CTCCTCCTCCTCCTCCTCCT-3′ (forward) and 5′-TGCCTGCTGGGA-ATTGTAGT-3′ (reverse).

Statistical analysis. The statistical significance was analyzed by one-way factorial analysis of variance and multiple comparison test or Student's *t*-test using Statview ver 5 (SAS Institute Inc., Cary, NC, USA).

Results

Characterization of JAK2V617F-Ba/F3

While mock- and Wt-Ba/F3 did not grow at all and gradually died in the absence of IL-3, *JAK2V617F*-Ba/F3 acquired the ability to proliferate in an IL-3-independent manner (Supplementary Fig. 1a). When *JAK2V617F*-Ba/F3 was cultured with JAK Inhibitor I, IL-3-independent proliferation and cell viability of *JAK2V617F*-Ba/F3 were inhibited remarkably. *JAK2V617F* as well as *BCR/ABL-Ba/F3* activated JAK2, STAT3 and STAT5 (Supplementary Fig. 2).

Cell cycle regulator, p27^{Kip1}, was upregulated in mock- and Wt- but not in JAK2V617F-Ba/F3

We analyzed the expressions of various cell cycle regulatory proteins. After 48 h of IL-3 deprivation, the cell cycle inhibitor p27^{Kip1}

increased in mock- and Wt-Ba/F3, but remained low in *JAK2V617F*-Ba/F3. Conversely, $p21^{WAF1}$ slightly decreased unexpectedly in IL-3-deprived mock- and Wt-Ba/F3, whereas it remained nearly the same in *JAK2V617F*-Ba/F3 (Fig. 1A). However, the proteasome inhibitor lactacystin induced the $p27^{Kip1}$ protein of *JAK2V617F*-Ba/F3 (Fig. 1B). In our model, cyclin D1 and D3, another cell cycle regulators, did not seem to play a major role in *JAK2V617F*-Ba/F3.

Inhibition of $p27^{Kip2}$ by JAK2V617F through SKP2 activation

Reportedly, $p27^{Kip1}$ was regulated by protein degradation rather than by transcriptional activation [16]. Recently, the relationship between $p27^{Kip1}$ and BCR/ABL has been reported using *BCR/ABL* stable transfectants [7]. Fig. 1A showed that Skp2 was decreased by IL-3 depletion in mock- and Wt-Ba/F3 but not in *JAK2V617F*-Ba/F3 cells. We also examined $p27^{Kip1}$ protein expression of *BCR/ABL*-, *STAT5 1*6* (constitutive active)- and *STAT3C* (constitutively active)-Ba/F3 cells with or without IL-3. Results showed that increase of $p27^{Kip1}$ protein induced with IL-3 deprivation was observed in wild type-*STAT* stable transfectants but not in constitutively active counterparts (Fig. 1C). Similarly, *BCR/ABL*-Ba/F3 showed high Skp2 protein in IL-3 depleted culture (Fig. 1D).

Skp2 mRNA level after IL-3 depletion

Fig. 2 illustrates semi-quantitative RT-PCR assay of $p27^{Kip1}$ and *Skp2* mRNAs in mock-, *JAK2V617F*-, *MIG*-mock- (as the control of *BCR/ABL* transfectant) and *BCR/ABL*-Ba/F3 cells. Semi-quantitative RT-PCR of $p27^{Kip1}$ mRNA showed that the $p27^{Kip1}$ message did not change significantly regardless of IL-3 presence. Therefore, the regulation of $p27^{Kip1}$ protein may not have taken place at the transcription level, although our preliminary promoter assay of $p27^{Kip1}$ revealed that the region between −348 and −2 bp was responsible for $p27^{Kip1}$ gene expression. On the contrary, it clearly shows that IL-3 depletion reduced *Skp2* mRNA in mock-cells, whereas *JAK2V617F*- and *BCR/ABL*-Ba/F3 did not show a similar decrease of mRNA, suggesting that maintaining *Skp2* mRNA level in *JAK2V617F*- and *BCR/ABL*-Ba/F3 cells is important for inhibiting the increase of the $p27^{Kip1}$ protein level after IL-3 depletion.

Determination of STAT site of Skp2 promoter

Next, we examined *Skp2* promoter analysis using a luciferase vector containing the 5′-promoter of *Skp2*. Fig. 3 shows the results of IL-3 depleted *JAK2V7617F*- and *BCR/ABL*-Ba/F3 cells. It suggests that a STAT motif located between −167 and −116 bp from the first exon (the second distal one in the inlet of Fig. 3A) was responsible for *JAK2V617F* as well as *BCR/ABL*.

EMSA and Chip assay of Skp2

Fig. 4 illustrates the results of EMSA. Among three bands observed, band *a* was produced with the wild type-oligo probe but not with the mutated-oligo in *JAK2V617F*-Ba/F3, and was erased with the cold competitor, suggesting its specificity (Fig. 4B and C). The intensity of band *c* is variable among experiments. Supershift assay using anti-STAT3 and anti-STAT5 antibody showed that anti-STAT5 antibody produced a supershifted band (Fig. 4D, asterisk). Anti-STAT3 antibody did not reduce band *a*. ChIP assay (Fig. 4E) suggests that STAT5 but not STAT3 was bound to the STAT site of the 5′-promoter, which is consistent with our EMSA.

Discussion

JAK2 is a member of the signal transducers located downstream of cellular receptors such as EPO receptor, IL-3 receptor, and GM-CSF receptor, and plays a critical role in the proliferation and differentiation of hematopoietic cells [17]. Recent studies revealed that a

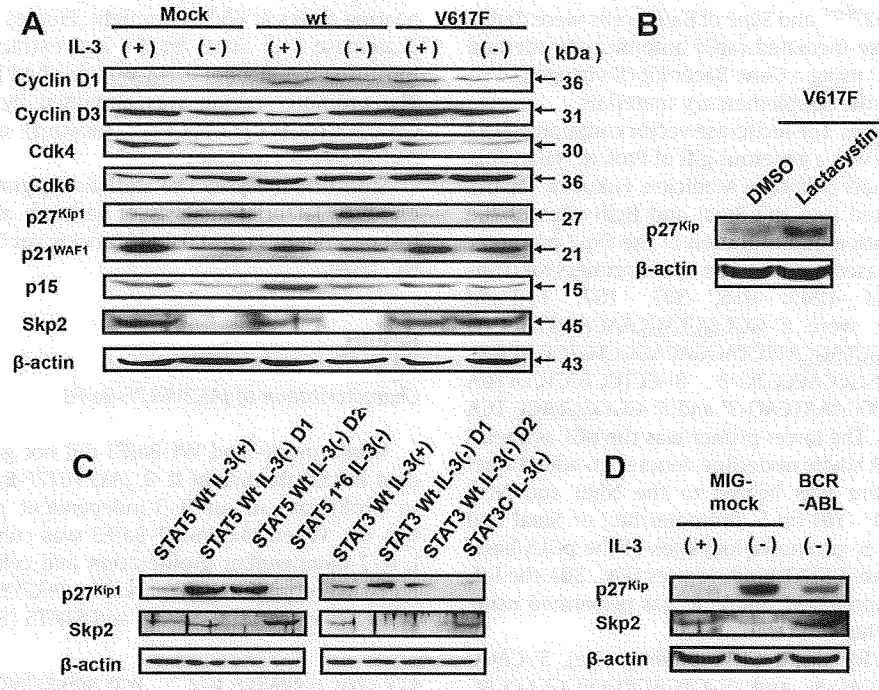

Fig. 1. Change of cell cycle regulators and related proteins in mock-, Wt- and *JAK2V617F*-Ba/F3 cells. (A) Protein levels of several cell cycle regulatory proteins including Cyclin D1, Cyclin D3, Cdk4, Cdk6, $p27^{kip1}$, $p21^{WAF1}$, p15 and SKP2 in mock-, Wt- and *JAK2V617F*-Ba/F3 were measured after 48 h with (+) or without (−) IL-3 addition. β-Actin was shown as the internal control. (B) The effect of lactacystin on P27 expression was examined using *JAK2V617F*-Ba/F3 cells. DMSO-treated cells were used as the vehicle control. (C) Stably overexpressed wild-type *STAT3*-, *STAT5*- and constitutive *STAT3* (*STAT3C*)-, and constitutive *STAT5* (*Stat5 1*6*)-Ba/F3 cells were examined for their p27 and Skp2 expression with or without IL-3 by Western blotting. (D) Using MIG-mock- and *BCR/ABL*-Ba/F3 cells, protein levels of p27 and Skp2 were analyzed by Western blotting.

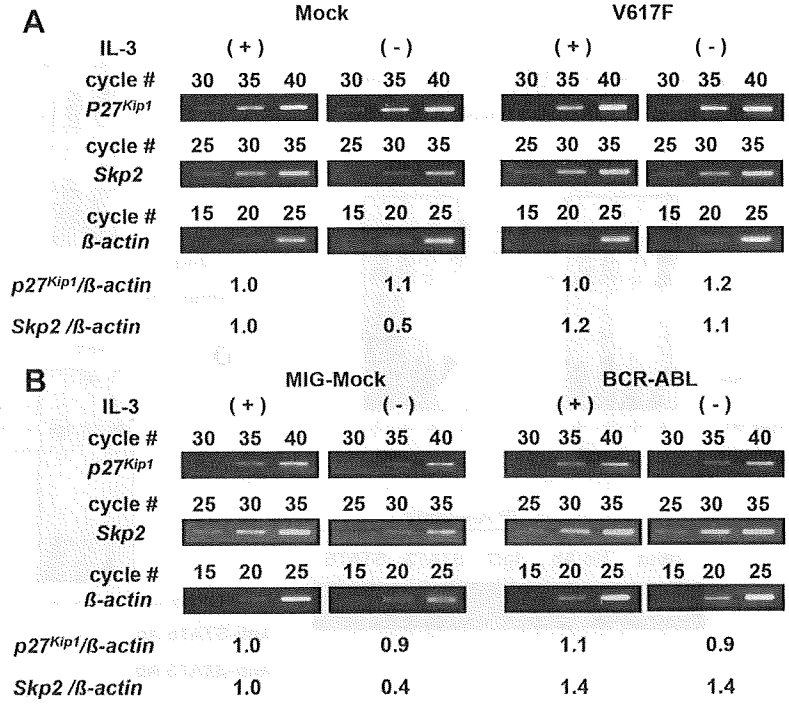

Fig. 2. *p27*^{Kip1} and *Skp2* mRNA expression of *JAK2V617F*-Ba/F3 and *BCR/ABL*-Ba/F3. *p27*^{Kip1} and *Skp2* mRNAs of mock-, *JAK2V617F*- and *BCR/ABL*-Ba/F3 were measured by the semi-quantitative RT-PCR method as described in Materials and methods. IL-3 (+) and IL-3 (−) denote cells cultured with or without Il-3 for 24 h. The relative expression level was calculated as the respective mRNA/β-actin mRNA. IL-3 treated culture was regarded as 1.0, respectively. A typical result was shown among several similar experiments.

high proportion of myeloproliferative disorder patients harbor a unique point mutation of *JAK2* [1,18]. It is also known that *JAK2V617F* mutation causes a gain of function of the JH2 domain, which inhibits the overactivation of the JH1 domain and controls its function [19]. This activated kinase might induce the change in the gene expression profile, which leads to the growth advantage of the MPD clone.

In the current study, we tried to elucidate the aberrant expression mechanism of the target protein of *JAK2V617F*. The p27^{Kip1} protein of *JAK2V617F*-Ba/F3 did not increase in the confluent state or cytokine depletion, whereas it increased in mock-Ba/F3, suggesting that *JAK2V617F* affects the cellular mechanism maintaining a normal cell growth character. However, increased p27^{Kip1} protein of *JAK2V617F*-Ba/F3 cells treated with a proteasome inhibitor,

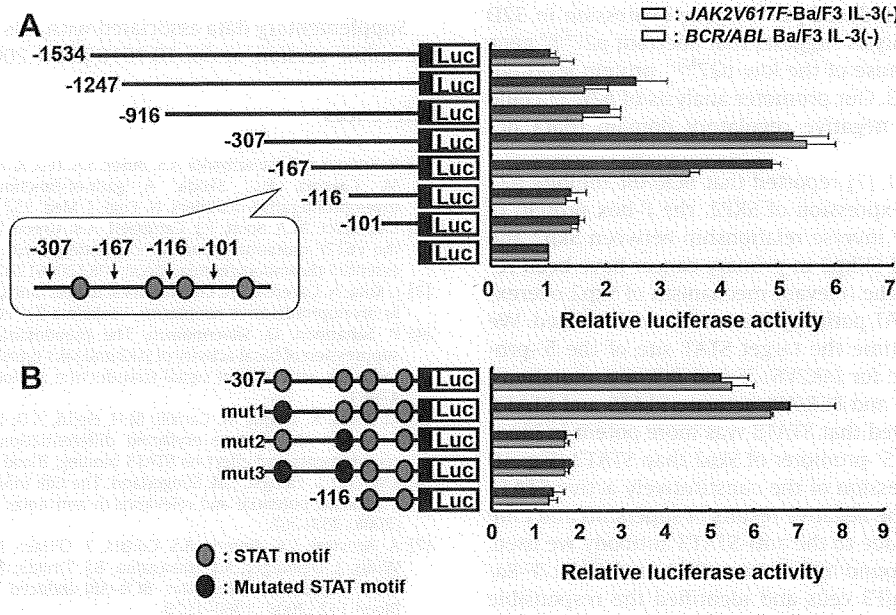

Fig. 3. Promoter analysis of *Skp2*. (A) Using *JAK2V617F*-Ba/F3 and *BCR/ABL*-Ba/F3 cells, the promoter activity of *Skp2* gene was analyzed by transfecting a pGL3 basic reporter containing various lengths of the 5′-promoter region as described in Materials and methods. Results were shown as the ratio of luciferase/β-gal. Data of pGL3 without insert was regarded as 1.0. Four STAT motifs located upstream of the first exon were illustrated on the left (solid gray circles). (B) The same experiments were carried out using mutated luciferase vectors. Solid grey and black circles denote wild and mutated STAT motif, respectively. The mean± was calculated from three independent experiments.

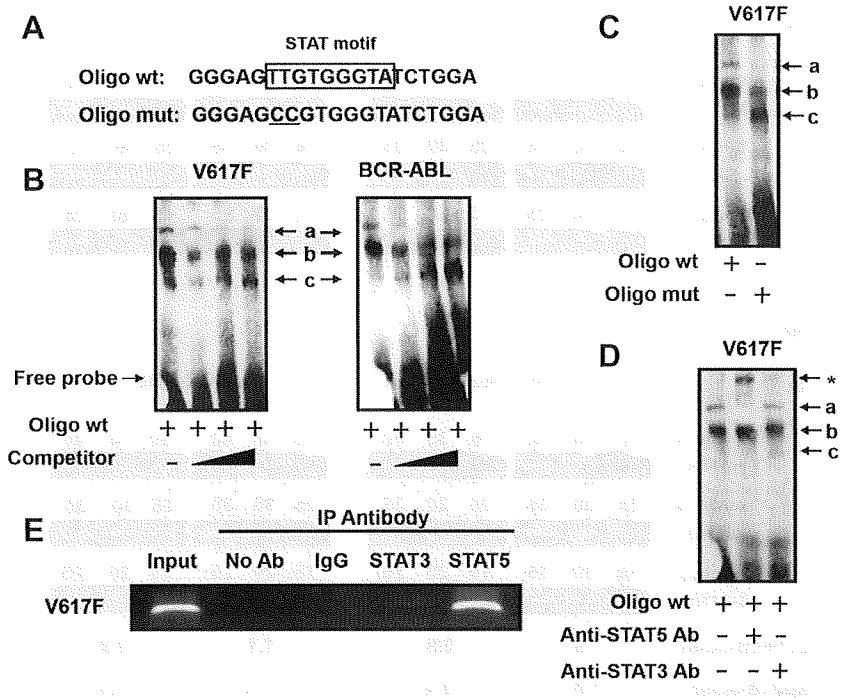

Fig. 4. EMSA and ChIP assay of *Skp2*. (A) Wild-type and mutated-oligoprobes used for EMSA of *Skp2* were shown as Oligo wt and Oligo mut. The STAT binding motif was boxed. Mutated bases are underlined. (B) EMSA was performed using *JAK2V617F*-Ba/F3 and *BCR/ABL*-Ba/F3 nuclear extracts, three bands (A–C) were observed. Cold competitor (5–20×) was added as indicated. (C) EMSA was performed using biotin-labeled wild or mutated-oligos shown in (A). (D) Anti-STAT3 and anti-STAT5 antibodies were used for the supershift assay according to Materials and methods. Shifted band was indicated by an asterisk. (E) ChIP assay was performed using anti-STAT3, anti-STAT5 antibody, normal mouse IgG and no antibody addition. Nuclear extract was prepared from *JAK2V617F*-Ba/F3 cells. A PCR primer set was described in Materials and methods.

lactacystin, suggests that $p27^{Kip1}$-related cell cycle regulation was not totally out of order in *JAK2V617F*-Ba/F3. On the contrary, $p21^{WAF1}$ of Ba/F3 cells might not be the major cell cycle regulator as suggested by others [20].

In colon carcinoma tumors and cell lines, Lin et al. reported the silencing of the JAK3/STAT3-induced increase in $p21^{WAF1}$ and $p27^{Kip1}$, suggesting that the JAK/STAT pathway inhibits these cell cycle regulators [21]. Although a STAT binding site is reported to be responsible for G-CSF-induced $p27^{Kip1}$ gene expression in 32D cells [22], our RT-PCR analysis suggests that aberrant $p27^{Kip1}$ gene expression was not the cause of the low $p27^{Kip1}$ protein level observed in *JAK2V617F*-Ba/F3. Our promoter analysis of $p27^{Kip1}$ could not detect any possible negative regulatory domain (data not shown).

Recently, Agarwal et al. [7] reported that *BCR/ABL* inhibits p27 expression through overexpression of SKP2, the F-box protein of the E3 ligase SCF^{SKP2}. The inverse relationship between Skp2 and p27 and oncogenic role of SKkp2 in primary cancer have been reported [23,24]. However, the relevant mechanism of Skp2 overexpresion by JAK2V617F/STAT pathway has not been elucidated. We determined for the first time the target STAT site of the 5′-promoter of *Skp2* responsible for *JAK2V617F* and *BCR/ABL* mutations. Although both *JAK2V617F* and *BCR/ABL* activate STAT5 and STAT3, our current analysis showed that STAT5 was more potent in binding to STAT motifs of the 5′-promoter of *Skp2* than STAT3 (Fig. 4D and E). Because overexpression of the constitutively active STAT3 could mimic *JAK2V617F*, the negative results of supershift and ChIP shown in Fig. 4 might be due to the anti-STAT3 antibody we used.

Furthermore, we also found high *Bcl-x_L* mRNA in *JAK2V617F*-Ba/F3 as well as *BCR/ABL*-Ba/F3 cells and identified the responsible STAT motif in the 5′-promoter of *Bcl-x_L* (data not shown). This may also contribute the pathogenesis of *JAK2V617F* mutation and of *BCR/ABL* translocation. Taken together, in the current study, the defective $p27^{Kip1}$ expression in response to cytokine depletion was characterized in *JAK2V617F*-Ba/F3 as well as *BCR/ABL*-Ba/F3. It is demonstrated a cell cycle regulator of $p27^{Kip1}$, and an E3 ligase subunit of Skp2, are finely controlled in Ba/F3 cells, but not in the presence of *JAK2V617F*. Future control of this particular pathway could help MPD as well as CML therapies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.04.015.

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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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KEY WORDS: Hepcidin, Bacterial infection, Allogeneic stem cell transplantation

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).

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 ($P < .001$).

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.

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.

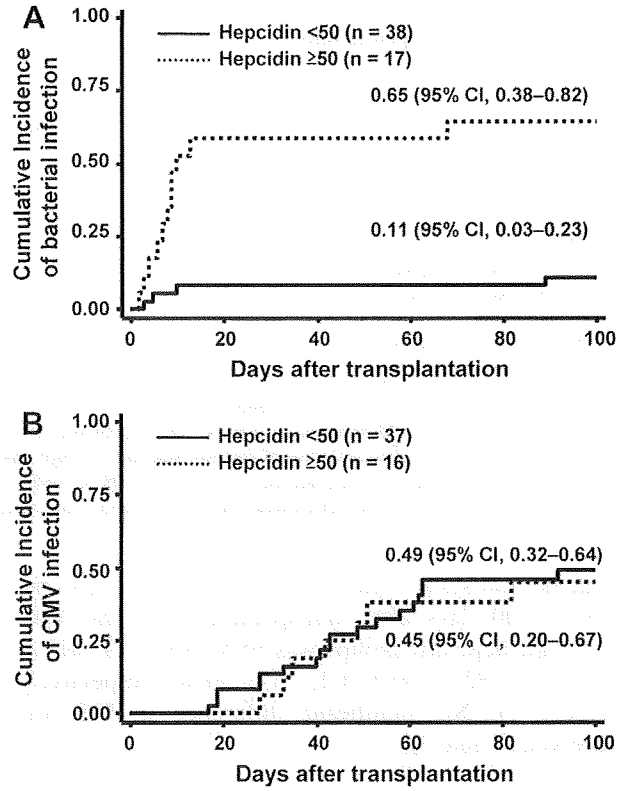

Figure 1. The cumulative incidences of documented bacterial infection (A) and cytomegalovirus (CMV) infection (B) at 100 days after stem cell transplantation. Solid black line, the low-hepcidin group (<50 ng/mL); solid gray line, the high-hepcidin group (≥50 ng/mL); CI, confidence interval. CMV infection 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.

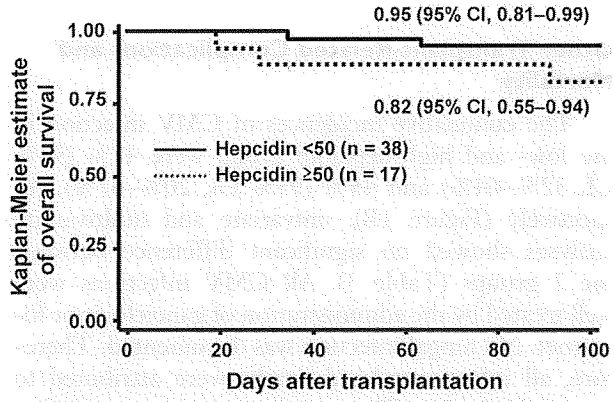

Figure 2. Kaplan-Meier estimate of OS at 100 days after stem cell transplantation. Solid black line, the low-hepcidin group (<50 ng/mL); solid gray line, the high-hepcidin group (≥50 ng/mL); CI, confidence interval.

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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Influence of Pretransplantation Serum Ferritin on Nonrelapse Mortality after Myeloablative and Nonmyeloablative Allogeneic Hematopoietic Stem Cell Transplantation

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Iron overload might be an important contributor to nonrelapse mortality (NRM) in hematopoietic stem cell transplantation (HSCT). We studied 264 patients undergoing allogeneic HSCT for hematologic malignancies between 1996 and 2006, using pretransplantation serum ferritin as a surrogate marker of iron overload. At 5 years, patients in the high ferritin group (≥599 ng/mL) had a lower overall survival (OS; 33.0% versus 63.5%; $P < .001$) and a higher NRM (34.9% versus 13.7%; $P < .001$) than those in the low ferritin group (<599 ng/mL). Multivariate analyses showed that high pretransplantation serum ferritin was a significant risk factor for worse survival (relative risk [RR] = 1.68; $P = .05$) and increased NRM (RR = 2.47; $P = .01$). There was no significant difference in the cumulative incidence of relapse, and acute and chronic graft-versus-host disease (aGVHD, cGVHD) between the 2 groups. Patients in the high ferritin group were more likely to die of infection ($P < .010$) and organ failure ($P < .019$). Similar results were observed after dividing the patients according to the intensity of conditioning regimens. These findings emphasize the prognostic impact of pretransplantation serum ferritin in HSCT recipients.

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KEY WORDS: Iron overload, Nonrelapse mortality, Hematopoietic stem cell transplantation, Ferritin, Hematologic malignancy, Hematopoietic cell transplantation-specific comorbidity index

INTRODUCTION

Iron overload is common in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) for hematologic disorders [1-15]. Several studies have reported the association between iron overload and transplant complications such as chronic liver disease [4-7,13,14,16-18], sinusoidal obstruction syndrome [13,14,19], infection [11-14,20-25], and idiopathic pneumonia syndrome [13]. The adverse impact of iron overload on survival of patients undergoing allogeneic HSCT for thalassemia is well established [26]. However, very few studies have assessed the role of iron overload in the outcome of allogeneic HSCT for other hematologic disorders. Previous studies showed that an elevated pretransplantation serum ferritin level, a surrogate marker of iron overload, was associated with lower overall survival (OS) and increased nonrelapse mortality (NRM) in patients undergoing myeloablative allogeneic HSCT for hematologic malignancies [9,10]. In general, the main causes of NRM after allogeneic HSCT are infection and graft-versus-host disease (GVHD). However, little is known about the causes of NRM, and the incidence of acute and chronic GVHD (aGVHD, cGVHD) in these patients.

Nonmyeloablative HSCT represents an effective strategy to reduce the toxicity of transplantation, as was shown in patients with older age or organ dysfunction with hematologic malignancies [27]. However, it is not clear whether the adverse impact of iron overload is present in nonmyeloablative HSCT.

The aims of this study were: (1) to determine the impact of pretransplantation serum ferritin on transplant outcome in patients undergoing allogeneic HSCT for hematologic malignancies; (2) to identify the causes of NRM in patients with elevated ferritin values; (3) to clarify the incidence of aGVHD and

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cGVHD in patients with high ferritin values, and (4) to examine whether this association of pretransplantation serum ferritin and transplant outcome is present in nonmyeloablative HSCT.

METHODS

Patients

We retrospectively studied 272 consecutive adult patients (≥16 years old) with hematologic malignancies who underwent allogeneic HSCT at the University of Tokyo Hospital, Japan, between June 1996 and October 2006. Two hundred sixty-four patients were included in the present study, as 8 patients were excluded because of the lack of data.

Myeloablative conditioning regimens included either total body irradiation (TBI)-based regimens or non-TBI-based regimens. TBI-based regimens mainly consisted of cyclophosphamide (Cy; 60 mg/kg/day for 2 days) plus fractionated TBI (12 Gy), whereas non-TBI-based regimens busulfan (Bu; 1 mg/kg every 6 hours for 4 days) plus Cy (60 mg/kg/day for 2 days). Patients were offered nonmyeloablative conditioning regimens as they were ineligible for conventional HSCT because of (1) age ≥56 years (n = 24); (2) presence of preexisting significant medical problem (severe cardiac dysfunction [n = 3], serious respiratory failure [n = 2], invasive pulmonary aspergillosis [n = 1]); and (3) history of high-dose HSCT (n = 10; 4 patients were also old, and 2 patients also had significant medical problems). Nonmyeloablative conditioning regimens consisted of fludarabine (Flu)-based regimens with or without low-dose TBI (4 Gy).

Prophylaxis for GVHD was performed with calcineurin inhibitors (cyclosporine [CsA] or tacrolimus) with or without short-term methotrexate (sMTX) in most patients. In vivo T cell depletion using alemtuzumab was performed in 30 patients, concomitant with CsA and sMTX.

Human leukocyte antigen (HLA)-matching for donor selection was based on serologic typing for HLA-A and -B antigens, and molecular typing for HLA-DRB1 antigen.

Disease morphology was determined according to the French-American-British classification. Standard-risk diseases were defined as acute leukemia in the first or second complete remission, chronic myelogenous leukemia (CML) in the first or second chronic phase, chemosensitive lymphoma, and myelodysplastic syndrome (MDS) in refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS). All the other conditions were classified as high-risk diseases.

Prophylaxis against bacterial infection was performed with tosufloxacin, fungal infection with fluconazole, herpes simplex virus (HSV) infection with acyclovir, and *Pneumocystis jiroveci* infection with sulfamethoxazole/trimethoprim.

Pretransplantation comorbidities were assessed retrospectively by comprehensive review of medical records and computer database system according to the hematopoietic cell transplantation-specific comorbidity index (HCT-CI) classification (44 missing data of pulmonary diffusing capacity for carbon monoxide and 3 missing data of whole pulmonary function tests) [28]. Each patient was assigned a comorbidity score and was stratified into low-risk (score 0), intermediate-risk (score 1-2), and high-risk (score ≥3) groups based on their total score.

Information concerning Karnofsky performance status was collected by a comprehensive review of medical records and assessed as per the Karnofsky scale. Karnofsky performance status scores were categorized as low (≤80%) or high (>80%) in accordance with previous reports [29].

Cause of death information was obtained from detailed review of the patients' medical charts and database that routinely record the primary cause of death as assigned by the treating physician at the time of patient death in a uniform manner.

This study was performed in accordance with the Helsinki Declaration and approved by the Ethics Committee of the University of Tokyo Hospital. All patients provided written informed consent.

Assessment of Iron Overload

We used pretransplantation serum ferritin as a surrogate marker of iron overload at the time of transplantation, because it is inexpensive, correlated with directly measured liver iron content [30], and reliable, with extensive clinical validation in monitoring iron status [31]. Serum ferritin was routinely measured as a part of routine pretransplant workup before the beginning of the conditioning regimen by using fluorescein enzyme immunoassay at our hospital. Because ferritin reflects acute inflammatory reaction besides iron overload, we included pretransplantation serum CRP and albumin, which were measured at the same time as serum ferritin and available in all of the studied patients, in the multivariate models for adjusting the influence of inflammation.

Statistical Methods

The duration of follow-up was the time from transplant to the last assessment for survivors. Variables related to the patients, the underlying diseases, the transplantation procedures, and the causes of NRM were compared between the groups with the Fisher's exact test for categoric variables and the Mann-Whitney *U*-test for continuous variables [32]. Probabilities of OS were calculated with the Kaplan-Meier method; the log-rank test was used for univariate comparisons [33]. For analyses of OS, death from any cause was considered an event, and data on

patients who were alive at the last follow-up were censored. Probabilities of NRM, relapse, and aGVHD and cGVHD were calculated with the use of the cumulative-incidence-function method [34]. For NRM, relapse was the competing event; for relapse, NRM was the competing event; for GVHD, death without GVHD was the competing event. Data on patients who were alive without an event were censored at the last follow-up.

The association of pretransplantation serum ferritin and the transplant outcomes was evaluated in multivariate analyses, with the use of Cox proportional-hazards regression to adjust for OS and Fine and Gray's proportional-hazards model for subdistribution of a competing risk for NRM [35]. The variables considered in multivariate analyses were pretransplantation serum ferritin, CRP, and albumin, age and sex of recipients, type of hematologic malignancies, disease status at transplantation, interval between diagnosis and transplantation, type of grafts, HLA compatibility, conditioning regimen, prophylaxis against GVHD, year of transplantation, history of prior transplantation, donor-recipient sex mismatch, HCT-CI, and KPS. Pretransplantation serum ferritin was coded according to whether it exceeds the median values. Pretransplantation serum CRP and albumin were coded according to whether they were normal or not. The relative risk estimates with their 95% confidence intervals and respective P values were reported from these analyses. All P values are 2-sided, with a type I error rate fixed at 0.05. Statistical analyses were performed with R 2.6.1 software (The R Foundation for Statistical Computing, 2007).

RESULTS

Patients

We reviewed the records of 264 adult patients who underwent allogeneic HSCT for hematologic malignancies at our institution. The median value of pretransplantation serum ferritin was 599 ng/mL (range: 5-8128 ng/mL). There was a strong relationship between pretransplantation serum ferritin and OS (Figure 1). The 5-year OS for patients with pretransplantation ferritin in the first quartile (<182 ng/mL) was 62.9% (95% confidence interval [CI], 51.6%-76.8%); in the second quartile (182-599 ng/mL), 64.2% (95% CI, 52.2%-79.0%); in the third quartile (599-1178.5 ng/mL), 40.3% (95% CI, 29.4%-55.4%); and in the fourth quartile (>1178.5 ng/mL), 23.7% (95% CI, 13.7%-40.9%). Compared with patients in the first quartile, those in the third and fourth quartile had a significantly inferior OS ($P < .001$, each), but those in the second quartile did not ($P = .856$). The patients, thus, were divided into 2 groups (ie, high ferritin group and low ferritin group) according to the median value of pretransplantation ferritin (ie, 599 ng/mL).

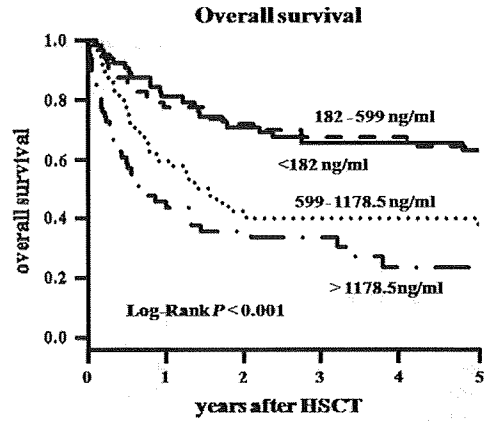

Figure 1. OS after allogeneic HSCT for hematologic malignancies in patients stratified by pretransplantation serum ferritin. *In the first quartile, ferritin level was <182 ng/mL, the second quartile, 182-599 ng/mL, the third quartile, 599-1178.5 ng/mL, and the fourth quartile, >1178.5 ng/mL. Compared with patients in the first quartile, those in the third and fourth quartile had significantly inferior survival ($P < .001$, each), but those in the second quartile did not ($P = .856$).

Their clinical characteristics are shown in Table 1. One hundred two patients received grafts from HLA-matched related donors, 77 patients from matched unrelated donors, and 85 patients from mismatched related (n = 45) or unrelated (n = 40) donors. Compared with patients in the low ferritin group, patients in the high ferritin group were more likely to be male, have acute myelogenous leukemia (AML) and MDS, have high-risk diseases at transplantation, have histories of prior transplantations, have high HCT-CI scores, and have low KPS. Patients in the high ferritin group were less likely to have CML and non-Hodgkin lymphoma (NHL), and have received CsA and sMTX as GVHD prophylaxis than those in the low ferritin group. Median CRP and albumin were 0.30 mg/dL (range: 0.02-17.20 mg/dL) and 4.0 g/dL (range: 2.3-5.2 g/dL), respectively. The percentage of patients with elevated serum CRP (>0.30 mg/dL) and lower albumin (<3.9 g/dL) in the high ferritin group was significantly higher than those in the low ferritin group.

OS, NRM, and Relapse

Median follow-up period for survivors after HSCT was 48.9 months (range: 3.0-136.7 months). At 5 years, patients in the high ferritin group had a significantly inferior OS than those in the low ferritin group (33.0% versus 63.5%; $P < .001$) (Figure 2A). Multivariate analyses of risk factors among all patients showed that high pretransplantation serum ferritin (relative risk [RR] = 1.68; 95% CI, 1.01-2.78; $P = .05$), high-risk disease (RR = 3.25; 95% CI, 1.96-5.39; $P < .01$), the use of alemtuzumab (RR = 3.41; 95% CI, 1.49-7.82; $P \leq .01$), and high-risk HCT-CI (RR = 1.75; 95%

Table 1. Clinical Characteristics of 264 Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation for Hematologic Malignancies According to Pretransplantation Serum Ferritin

Variable	Total (N = 264)	Low ferritin < 599 ng/mL (N = 132)	High ferritin ≥599 ng/mL (N = 132)	*P* Value*
Age, years				.082
Median	40	38	41	
Range	16-66	16-62	18-66	
Sex				.003
Male	170 (64)	73 (55)	97 (73)	
Female	94 (36)	59 (45)	35 (27)	
Disease classification				<.001
Acute myelogenous leukemia	78 (30)	16 (12)	62 (47)	
Acute lymphoblactic leukemia	62 (23)	31 (23)	31 (23)	
Chronic myelogenous leukemia	51 (19)	45 (34)	6 (5)	
Myelodysplastic syndrome	34 (13)	14 (11)	20 (15)	
RA/RARS	9 (3)	2 (2)	7 (5)	
RAEB/RAEBT	21 (8)	12 (9)	9 (7)	
CoL	4 (2)	0 (0)	4 (3)	
Non-Hodgkin lymphoma	33 (13)	24 (18)	9 (7)	
Others	6 (2)	2 (2)	4 (3)	
Disease risk at transplantation†				<.001
Standard	146 (55)	88 (67)	58 (44)	
High	118 (45)	44 (33)	74 (56)	
Interval between diagnosis and transplantation, months				1.000
Median	10.6	10.9	10.1	
Range	1.8-202.3	2.4-173.4	1.8-202.3	
Graft source				.510
Bone marrow	168 (64)	89 (67)	79 (60)	
Peripheral blood	85 (32)	39 (30)	46 (35)	
Others	11 (4)	4 (3)	7 (5)	
Donor type				.348
Matched related donor	102 (39)	53 (40)	49 (37)	
Matched unrelated donor	77 (29)	42 (32)	35 (27)	
Mismatched donor	85 (32)	37 (28)	48 (36)	
Conditioning regimen				.099
Myeloablative	230 (87)	121 (92)	109 (82)	
TBI-based regimen	190 (72)	97 (73)	93 (70)	
Non-TBI-based regimen	40 (15)	24 (18)	16 (12)	
Nonmyeloablative	34 (13)	11 (8)	23 (18)	
TBI-based regimen	12 (5)	3 (2)	9 (7)	
Non-TBI-based regimen	22 (8)	8 (6)	14 (11)	
GVHD prophylaxis				.028
Cyclosporine alone	11 (4)	2 (2)	9 (7)	
Cyclosporine and methotrexate	201 (76)	110 (83)	91 (69)	
Tacrolimus and methotrexate	24 (9)	9 (7)	15 (11)	
Alemzutumab	28 (11)	11 (8)	17 (13)	
Transplant year				.122
1995-1998	43 (16)	25 (19)	18 (14)	
1999-2002	113 (43)	61 (46)	52 (39)	
2003-2006	108 (41)	46 (35)	62 (47)	
Prior transplantation				.005
	243 (92)	128 (97)	115 (87)	
>1	21 (8)	4 (3)	17 (13)	
Sex pair (Donor-Recipient)				.396
Female-male	67 (25)	30 (23)	37 (28)	
Others	197 (75)	102 (77)	95 (72)	
Hematopoietic cell transplantation specific comorbidity index				<.001
Low (0)	84 (32)	54 (41)	30 (23)	
Intermediate (1-2)	96 (36)	51 (39)	45 (34)	
High (≥)	84 (32)	27 (20)	57 (43)	
Karnofsky performance status				.001
>80	168 (64)	97 (73)	71 (54)	
≤80	96 (36)	35 (27)	61 (46)	
C-reactive protein				<.001
03	170 (64)	105 (80)	65 (49)	
>0.3	94 (36)	27 (20)	67 (51)	
Albumin				<.001
≥3.9	178 (67)	104 (79)	74 (56)	
<3.9	86 (33)	28 (21)	58 (44)	

RA indicates refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEBT, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia; TBI, total body irradiation; GVHD, graft-versus host disease.
*The Fisher's exact test was used for categoric variables and the Mann-Whitney *U*-test for continuous variables.
†Standard-risk diseases were defined as acute leukemia in the first or second complete remission, chronic myelogenous leukemiin the first or second chronic phase, chemosensitive lymphoma, and myelodysplastic syndrome in refractory anemia or refractory anemia with ringed sideroblasts. All the other conditions were classified as high-risk diseases.

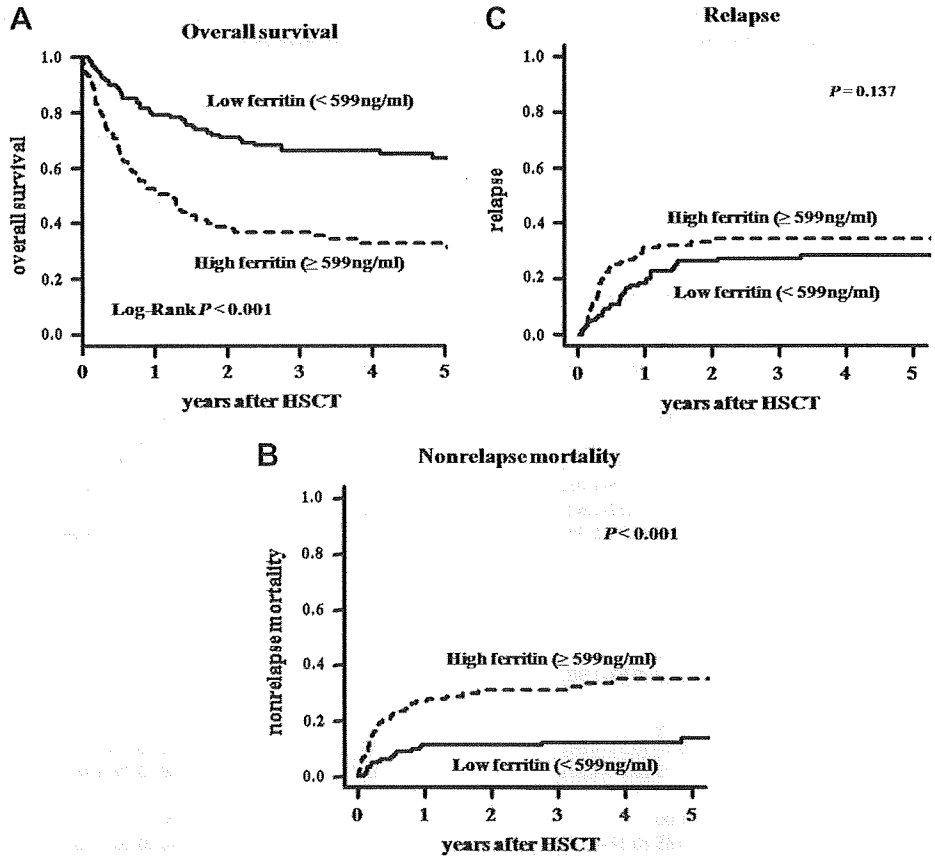

Figure 2. OS (A), NRM (B), and relapse (C) rates after allogeneic HSCT for hematologic malignancies in patients divided by the median value of the pretransplantation serum ferritin level. Patients in the high ferritin group had significantly lower OS and higher NRM than those in the low ferritin group ($P < .001$, each). The relapse rate was similar between the 2 groups ($P = .137$).

CI, 1.02-2.99; $P = .04$) significantly predicted worse OS (Table 2).

At 5 years, patients in the high ferritin group had a significantly higher NRM than those in the low ferritin group (34.9% versus 13.7%; $P < .001$) (Figure 2B). There was no statistical difference in relapse rate between the 2 groups of patients (34.4% versus 28.3%; $P = .137$) (Figure 2C). In multivariate analyses, high pretransplantation serum ferritin (RR = 2.47; 95% CI, 1.21-5.07; $P = .01$), the use of alemtuzumab (RR = 4.70; 95% CI, 1.19-18.58; $P = .03$), and intermediate-risk (RR = 4.04; 95% CI, 1.57-10.37; $P < .01$) and high-risk HCT-CI (RR = 4.34; 95% CI, 1.64-11.52; $P < .01$) were the significant risk factors associated with increased NRM (Table 2).

aGVHD and cGVHD

The cumulative incidence of grade II, III, and IV aGVHD was similar between the 2 groups of patients (37.1% versus 42.4% for the patients in the high and low ferritin group; $P = .422$). There was no statistical difference in the incidence of extensive cGVHD between the 2 groups of patients (43.9% versus 40.4%; $P = .703$).

Causes of NRM

A total of 58 patients died from nonrelapse causes, 16 of 132 patients in the low ferritin group (12%) and 42 of 132 patients in the high ferritin group (32%). Table 3 lists the causes of NRM according to pretransplantation serum ferritin. Patients in the high ferritin group were more likely to die of infection ($P = .010$) and organ failure ($P = .019$), compared with those in the low ferritin group.

Outcomes after Nonmyeloablative Compared with Myeloablative Conditioning

As the decreased OS was attributable to the increased NRM in patients with elevated ferritin values who underwent allogeneic HSCT for hematologic malignancies, we aimed to investigate whether this relationship is still present in nonmyeloablative HSCT. After myeloablative HSCT, patients in the high ferritin group had an inferior OS (38.5% versus 62.6%; $P < .001$), and a higher NRM (30.8% versus 13.8%; $P < .001$) at 5 years, compared with those in the low ferritin group. However, there was no significant difference in relapse rate (33.2% and 28.8%; $P = .302$) between the 2 groups. Similarly, after nonmyeloablative HSCT,

Table 2. Factors Associated with Overall Survival and Nonrelapse Mortality within 264 Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation for Hematologic Malignancies: Multivariate Analysis

Variable	Overall Survival		Nonrelapse Mortality	
	RR (95%CI)	*P* Value*	RR (95%CI)	*P* Value*
Ferritin				
Low (<599 ng/mL)	1.00		1.00	
High (≥599 ng/mL)	1.68 (1.01-2.78)	.05	2.47 (1.21-5.07)	.01
Age				
<50 years	1.00		1.00	
≥50 years	0.97 (0.55-1.69)	.90	1.35 (0.56-3.23)	.51
Sex				
Male	1.00		1.00	
Female	1.30 (0.78-2.14)	.31	1.30 (0.54-3.10)	.56
Disease classification				
Acute myelogenous leukemia	1.00		1.00	
Acute lymphoblastic leukemia	1.36 (0.78-2.36)	.28	0.90 (0.38-2.18)	.82
Chronic myelogenous leukemia	0.90 (0.46-1.78)	.77	1.84 (0.70-4.84)	.22
Myelodysplastic syndrome	0.56 (0.29-1.06)	.07	1.97 (0.77-5.01)	.15
Non-Hodgkin lymphoma	1.28 (0.62-2.62)	.50	2.22 (0.91-5.42)	.08
Others	1.35 (0.34-5.39)	.67	1.45 (0.11-19.04)	.78
Disease risk at transplantation				
Standard	1 00		1 00	
High	3.25 (1.96-5.39)	<.01	1.22 (0.61-2.44)	.58
Interval between diagnosis and transplantation				
≤10.6 months	1.00		1.00	
>10.6 months	1.28 (0.83-1.98)	.27	1.47 (0.75-2.80)	.26
Graft source				
Bone marrow	1.00		1.00	
Peripheral blood	0.97 (0.51-1.84)	.93	0.52 (0.18-1.50)	.23
Others	1.73 (0.71-4.23)	.23	1.88 (0.79-4.46)	.15
Donor type				
Matched related donor	1.00		1.00	
Alternative donor	0.65 (0.37-1.14)	.13	0.46 (0.20-1.05)	.06
Conditioning regimen				
Myeloablative	1.00		1.00	
Nonmyeloablative	1.13 (0.52-2.46)	.75	0.90 (0.29-2.82)	.86
Total body irradiation				
Non-TBI-based regimen	1.00		1.00	
TBI-based regimen	1.06 (0.60-1.85)	.84	1.68 (0.70-4.04)	.25
GVHD prophylaxis				
Cyclosporine-based	1.00		1.00	
Tacrolimus-based	1.02 (0.51-2.02)	.96	1.21 (0.37-3.92)	.75
Alemzutumab	3.41 (1.49-7.82)	<.01	4.70 (1.19-18.58)	.03
Transplant year				
1995-1998	1.00		1.00	
1999-2002	1.20 (0.66-2.19)	.55	1.10 (0.45-2.67)	.84
2003-2006	1.10 (0.54-2.22)	.80	0.76 (0.28-2.09)	.59
Prior transplantation				
0	1.00		1.00	
≥1	1.61 (0.85-3.06)	.15	1.62 (0.53-4.96)	.40
Sex pair (Donor-Recipient)				
Female-male	1.00		1.00	
Others	1.03 (0.65-1.63)	.91	0.96 (0.47-1.97)	.92
Hematopoietic cell transplantation specific comorbidity index				
Low (0)	1.00		1.00	
Intermediate (1-2)	1.71 (1.00-2.91)	.05	4.04 (1.57-10.37)	<.01
High (≥3)	1.75 (1.02-2.99)	.04	4.34 (1.64-11.52)	<.01
Karnofsky performance status				
>80	1.00		1.00	
≤80	0.89 (0.56-1.40)	.60	0.86 (0.43-1.71)	.66
C-reactive protein				
≤0.3	1.00		1.00	
>0.3	1.45 (0.94-2.23)	.10	1.88 (0.90-3.90)	.09
Albumin				
≥3.9	1.00		1.00	
<3.9	1.43 (0.90-2.28)	.13	1.36 (0.71-2.61)	.35

RR indicates relative risk; CI, confidence interval; GVHD, graft-versus host disease.

*Cox proportional-hazards regression model was used for overall survival, and Fine and Gray's proportional-hazards model for subdistribution of a competing risk was used for continuous nonrelapse mortality.

Table 3. Causes of Nonrelapse Death in 264 Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation for Hematologic Malignancies According to Pretransplantation Serum Ferritin

Variable	Total (N = 264)	Low ferritin <599 ng/mL (N = 132)	High ferritin ≥599 ng/mL (N = 132)	P Value†
	No. of Patients (%)*			
Nonrelapse mortality	58 (22)	16 (12)	42 (32)	
GVHD	14 (5)	5 (4)	9 (7)	.411
Infection‡	25 (9)	6 (5)	19 (14)	.010
Hemorrhage	2 (1)	1 (1)	1 (1)	1.000
Interstitial pneumonia	5 (2)	2 (2)	3 (2)	.663
Organ failure§	10 (4)	1 (1)	9 (7)	.019
Secondary malignancy	1 (0)	1 (1)	0 (0)	1.000
Unknown	1 (0)	0 (0)	1 (1)	1.000

GVHD indicates graft-versus host disease.

*Percentages of patients in each group are shown in the parentheses. Because of rounding, the percentages of respective nonrelapse causes of death may not sum to the percentage of overall nonrelapse mortality.

†The Fisher's exact test was used.

‡A comparable proportion of patients who died of infect, had grade II, III, and IV acute GVHD/or extensive chronic GVHD; 5 out of 6 in the low ferritin group and 11 out of 19 in the high ferritin group. Infections in the low ferritin group were bacterial (n = 1), fungal (n = 3), and viral (n = 2). Infections in the high ferritin group were bacterial (n = 10), fungal (n = 6), and viral (n = 3).

§Organ failure was assigned when the specific cause of organ failure, including infection, GVHD, hemorrhage, or veno-occlusive disease, is not determined despite the appropriate wsorerep. Multiorgan failure was assigned when two or more organ failure was attributable to death. Organ failure in the low ferritin group included heart failure (n = 1). Organ failure in the high ferritin group included heart failure (n = 2) renal failure (n = 3), hepatic failure (n = 2), and multiorgan failure (n = 2).

patients in the high ferritin group had an inferior OS (7.0% versus 80.8%; $P = .002$) (Figure 3A), and a higher NRM (54.3% versus 9.1%; $P < .001$) (Figure 3B) at 5 years, compared with those in the low ferritin group. However, there was no significant difference in relapse rates (39.1% versus 22.7%; $P = .137$) (Figure 3C) between the 2 groups. Thus, patients with elevated ferritin values had decreased OS because of increased NRM after allogeneic HSCT, regardless of the intensity of conditioning regimens.

DISCUSSION

The current analysis extended previous reports on the role of pretransplantation serum ferritin in allogeneic HSCT. In this study, we showed that an elevated pretransplantation serum ferritin level was associated with decreased OS and increased NRM in patients undergoing allogeneic HSCT for hematologic malignancies, consistent with previous observations [9,10]. Furthermore, to our knowledge, this is the first report to clarify the causes of NRM and the incidence of aGVHD and cGVHD in patients with high ferritin values. Moreover, we showed that pretransplantation serum ferritin affected adversely the transplant outcome as well in patients who received nonmyeloablative conditioning regimens.

Decreased OS in patients with high ferritin values was attributable to increased NRM. Among the various causes that might explain NRM, infection and organ failure were significantly more frequent in patients in the high ferritin group than those in the low ferritin group. These findings are consistent with the previous reports on the association between iron overload and transplant related complications, such as infection [11-14,20-25] and chronic liver disease [4-7,13,14,16-18] after HSCT. One explanation for these results is that free radical iron, in a state of iron excess, can lead to a pro-oxidant state by generating free radicals, which could subsequently cause tissue injury and increase the risk of transplant related complications [14]. Indeed, a previous study showed the presence of catalytic iron that increased oxidative damage to proteins in the serum of children with acute lymphoblastic leukemia (ALL) receiving high-dose MTX [36]. Furthermore, iron is an essential cofactor for the growth of a number of opportunistic bacteria and fungi and free iron can also increase the susceptibility of patients to infections through impairment of cellular immunity and inhibition of chemotaxis and phagocytosis [14].

We used the median value of pretransplantation serum ferritin level (ie, 599 ng/mL) as the cutoff value for dividing the patients into the 2 groups. Guidelines on iron-chelation therapy for heavily transfused patients such as thalassemia, aplastic anemia (AA), and MDS recommend that the patients with serum ferritin levels higher than 1000-2500 ng/mL might benefit from the treatment for iron overload [37-39]. We showed that patients with moderately elevated serum ferritin levels (599-1178.5 ng/mL) had inferior survival, compared with patients with normal (5-182 ng/mL) and mildly elevated serum ferritin levels (182-599 ng/mL) (Figure 1). This suggests that pretransplantation serum ferritin has adverse effects at lower level for HSCT recipients than transfused patients.

In patients undergoing allogeneic HSCT, the main cause of NRM in patients with high ferritin values was infection. This cause of death is quite different from that in chronically transfused patients such as thalassemia, in which iron overload associated cardiomyopathy and liver fibrosis were among the leading causes of death before the introduction of iron-chelation therapy [31]. Possible explanation for this difference is that patients undergoing allogeneic HSCT for hematologic malignancies were more susceptible to infections than those receiving chronic transfusions because of prolonged neutropenia and breaks in the mucocutaneous barrier because of the HSCT preparative regimens, cell-mediated and humoral immunity defects, and impaired functioning of the reticuloendothelial system [40]. Considering that

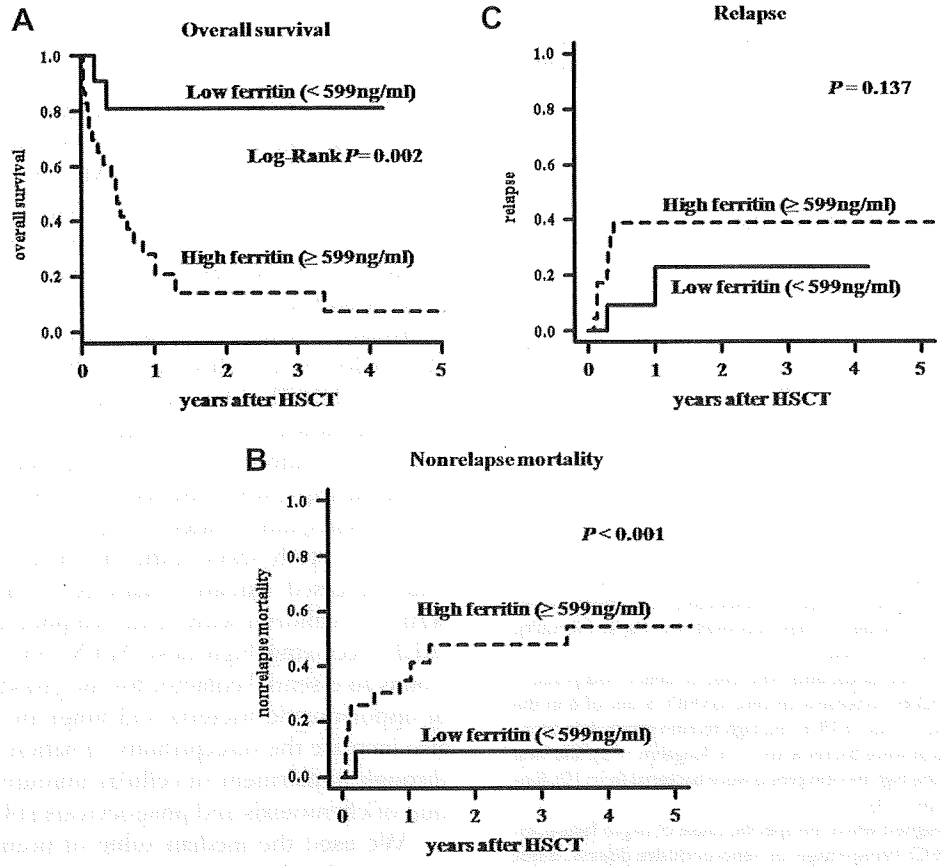

Figure 3. OS (A), NRM (B), and relapse (C) rates after allogeneic HSCT following nonmyeloablative conditioning in patients divided by the median value of the pretransplantation serum ferritin level. Patients in the high ferritin group had significantly lower OS ($P = .002$) and higher NRM ($P < .001$) than those in the low ferritin group. The relapse rate was similar between the 2 groups ($P = .137$).

the incidence of severe aGVHD and cGVHD in the high ferritin group was comparable with those in the low ferritin group, elevated ferritin value might be a risk factor for infection, independent of aGVHD and cGVHD. Thus, these results indicated the significance of the prophylaxis and surveillance regimen for infectious diseases to prevent iron overload related complications in patients with high ferritin values undergoing allogeneic HSCT.

Allogeneic HSCT following nonmyeloablative conditioning has been performed for hemoglobinopathies and reduced the transplant related complications in heavily transfused patients [41]. We, however, showed that the association of pretransplantation serum ferritin and NRM was present as well in patients undergoing nonmyeloablative HSCT. As patients with elevated ferritin value had decreased OS and increased NRM after allogeneic HSCT, regardless of the intensity of conditioning regimens, intervention for iron overload, including phlebotomy and iron-chelation therapy, in the pre- and posttransplantation setting may be important to reduce the morbidity and mortality in these patients, as well as thalassemia patients [42,43], and its feasibility and safety need to be investigated in further study.

This study had a number of limitations. (1) This study was a retrospective, single-institution study. In particular, it is difficult to assign, retrospectively, the causes of death in the posttransplantation setting. Additional prospective multicenter studies are required to confirm the true association between iron overload and transplant outcomes. (2) Ferritin is an acute phase reactant and is not the best indicator of iron overload. However, ferritin is a useful noninvasive surrogate, because it is inexpensive, widely available, and reliable with extensive clinical validation in monitoring iron status [31]. Furthermore, ferritin has a strong association with hepatic iron concentration directly measured by liver biopsy [30]. Moreover, several guidelines on iron overload recommend serum ferritin as a diagnostic tool of iron overload [37-39]. Although the influence of inflammation could be adjusted by the inclusion of CRP and/or albumin in the multivariate analyses, further studies using more reliable methods for assessment of body iron stores such as liver biopsy and magnetic resonance imaging would be helpful [15,18]. (3) The patients included in this study were a heterogeneous population who had various backgrounds and diseases, and underwent various transplant procedures, although these factors were adjusted in the multivariate analyses.