Prognostic impact of the dosage of methotrexate combined with tacrolimus for graft-versus-host disease prophylaxis after cord blood transplantation

Original Article

Title:

Prognostic impact of the dosage of methotrexate combined with tacrolimus for graft-versus-host disease prophylaxis after cord blood transplantation

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Running head: Dosage of MTX for GVHD prophylaxis in CBT

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Abstract

The optimal dosage of methotrexate (MTX) for graft-versus-host-disease (GVHD) prophylaxis after cord blood transplantation (CBT) has not been well elucidated. Therefore, we conducted a retrospective study comparing a mini-MTX group (5 mg/m^2 on day 1, 3 and 6) to a short-MTX group (10 mg/m^2 on day 1 and 7 mg/m^2 on day 3 and 6) after CBT. Sixty-three patients were classified as the mini-MTX group and 20 as the short-MTX group. The median time and cumulative incidence of neutrophil engraftment did not vary between the two groups. The cumulative incidence of grade 2–4 and grade 3–4 acute GVHD was similar in both groups. Overall survival in the mini-MTX group was significantly lower than in the short-MTX group (46.9% vs. 88.7% at 1 year, $p<0.01$), contributing to higher non-relapse mortality (NRM) in the mini-MTX group (32.0% vs. 5.0% at 1 year, $p=0.02$). In multivariate analysis, the mini-MTX regimen was the most powerful prognostic factor for OS (hazard ratio: 4.11; $p=0.03$). Although the reduced dosage of MTX had no effect on neutrophil engraftment, increased NRM due to higher incidence of infection, graft failure, and severe acute GVHD resulted in a lower survival rate in the mini-MTX group after CBT.

Keywords

GVHD prophylaxis, methotrexate, tacrolimus, cord blood transplantation

Introduction

Cord blood transplantation (CBT) has been established as an alternative source of hematopoietic stem cell transplantation (HSCT) [1-6]. Several studies have reported that the incidence of severe acute graft-versus-host-disease (GVHD) after CBT was similar to that following HLA 8/8 allele-matched unrelated bone marrow transplantation [6, 7]. Recently, Kanda et al reported that the overall survival (OS) in patients with grade 1–2 acute GVHD following CBT was significantly superior to that of patients with grade 3–4 acute GVHD or without acute GVHD, which contributed to the association between grade 1 or higher acute GVHD and lower relapse rate, and between grade 3–4 acute GVHD and a higher non-relapse mortality (NRM) rate [8]. Therefore, as with the use of other hematopoietic stem cell sources, the prevention of acute GVHD in patients who underwent CBT is considered to be important for the improvement of clinical outcomes.

The combination of calcineurin inhibitor (CNI) and short-term methotrexate (MTX) is considered as one of the standard regimens for GVHD prophylaxis after CBT, because of a lower incidence of acute GVHD with the combination compared with that with CNI alone [9-11]. On the other hand, since infection during bone marrow suppression until neutrophil engraftment is one of the main causes of NRM in patients who underwent CBT, whether the MTX dosage can affect the time to neutrophil engraftment needs to be understood. Although the MTX dosage for GVHD prophylaxis was reduced in several studies to avoid MTX-related toxicities, such as delayed neutrophil engraftment [12, 13], the optimal dosage of MTX in combination with CNI has not yet been well elucidated.

Here, we conducted a retrospective study to compare the mini-MTX regimen (MTX dose: 5 mg/m^2 on day 1, 3, and 6) with the short-MTX regimen (MTX dose: 10 mg/m^2 on day1 and 7 mg/m^2 on day 3 and 6) in order to determine the optimal dosage of MTX in combination with tacrolimus (TAC) for GVHD prophylaxis in CBT.

Patients and methods

Patients

Eighty-three adult patients who underwent a single-unit CBT at Hamamatsu University Hospital and Hamamatsu Medical Center between February 2006 and September 2018 were evaluated. CBT was selected for patients without HLA-identical siblings or suitable unrelated donors. The MTX dosage for GVHD prophylaxis was classified into the two groups; the mini-MTX group (5 mg/m^2 on day 1, 3 and 6) and the short-MTX group (10 mg/m^2 on day 1 and 7 mg/m^2 on day 3 and 6). The mini-MTX regimen was used between February 2006 and March 2016, and the short-MTX regimen after February 2016 in both the institutions. The study was approved by the Institutional Review Boards of each of the participating institutions. Informed consent was obtained from patients before registration in the study in accordance with the Declaration of Helsinki. All data were updated in January 2019.

Transplantation procedures and treatments

The selection of each cord blood unit was based on the total nucleated cell count being greater than 2.0×10^7 per recipient weight (a total nucleated cell count of less than 2.0×10^7 per recipient weight was permitted if the optimal cord blood unit was not available.) and HLA matching at the antigen level; which is permitted within a 2-loci mismatch among the HLA-A, -B and -DR loci. T cell depletion was not performed. The conditioning regimen was defined as myeloablative conditioning (MAC) if total-body irradiation $\geq$ 8 Gy, intravenous busulfan > 6.4 mg/kg or melphalan > 140 mg/m^2 was used; otherwise, it was defined as reduced-intensity conditioning (RIC) [14-16]. All of the patients received

granulocyte colony-stimulating factor from day 5. TAC was started on day –1 at a dose of 0.025 mg/kg/day by continuous infusion and the dose was adjusted to maintain a blood concentration of approximately 15 ng/ml. TAC was changed to an oral form when it could be tolerated by the patient at tripled doses. For patients without GVHD, we started to taper TAC from day 50 by 5% per week and discontinued the drugs at around day 180. MTX was administered using the mini-MTX or the short-MTX regimens. Prophylaxis against bacterial, fungal and *pneumocystis jirovecii* infections consisted of fluoroquinolones, azole antifungals, and sulfamethoxazole/trimethoprim. Acyclovir was given from day −7 to 35 for prophylaxis against herpes simplex and varicella zoster virus infections. Cytomegalovirus (CMV) antigenemia assays using C7 antibody were performed at least once a week after engraftment, and preemptive therapy with ganciclovir was initiated for high-risk patients [17]. Patients who developed grades 2–4 acute GVHD were treated with 1–2 mg/kg of (methyl-)prednisolone, whereas grade 2 acute GVHD, which was limited to the skin, was treated with a topical steroid with or without a hydrocortisone infusion.

Definitions

Standard-risk diseases included acute leukemia in complete remission (CR), chronic myeloid leukemia in the chronic phase, malignant lymphoma in CR, myelodysplastic syndrome in refractory anemia or refractory anemia with ring sideroblasts and non-malignant hematological diseases. High-risk diseases included all other diagnoses other than the standard-risk diseases.

Neutrophil engraftment was defined as an absolute neutrophil count of at least 500/μL at three consecutive time points. Platelet engraftment was defined as a platelet count of

20,000/μL without transfusion support. Graft failure was defined as: (1) the combination of neutrophil count <500/μL and marrow hypoplasia for >60 days of CBT with the existence of donor-type hematopoiesis (mixed or complete donor chimerism) or (2) the complete loss of donor-type hematopoiesis occurring at any time after transplantation[18]. The diagnosis and grading of acute and chronic GVHD were performed based on established criteria[19, 20]. Immune reactions were defined based on a previous report [21]. Briefly, when febrile patients (body temperature ≥38°C) with no evidence of infection or adverse effects from medication, exhibited skin eruption, diarrhea, jaundice (serum total bilirubin >2.0 mg/dl) or body weight gain >10% of the baseline, these changes were defined as immune reactions. Reactions were classified into subtypes of pre-, peri- and post-engraftment according to timing. Immune reactions developing ≥6 days before engraftment were defined as pre-engraftment immune reaction (PIR), whereas reactions within 5 days of engraftment were defined as engraftment syndrome (ES). NRM was defined as any death without the progression of an underlying disease. Relapse or progression disease (PD) was defined as recurrence or progression of the underlying hematological disease.

Statistical analysis

We compared and evaluated the endpoints between the mini-MTX and the short-MTX group. Evaluated endpoints were as follows: median time to neutrophil and platelet engraftment, the cumulative incidence of neutrophil and platelet engraftment, graft failure, acute and chronic GVHD, PIR and ES, relapse or PD, NRM, OS, and progression free survival (PFS). Fisher's exact test was used to compare the categorical variables and the Mann-Whitney U-test was used to compare the continuous variables. OS and PFS were

121 estimated according to the Kaplan-Meier method and compared among groups using the
122 log-rank test. The incidence of neutrophil and platelet engraftment, GVHD, PIR and ES,
123 relapse or PD, and NRM were compared using Gray's test. For the analysis of the
124 cumulative incidence of engraftment, PD and the all cause of death before engraftment
125 were defined as competing events. For the analysis of cumulative incidence of acute and
126 chronic GVHD, graft failure and death were the competing events; for NRM, death due
127 to primary disease was the competing event; and for relapse or PD, NRM was the
128 competing event. The threshold for significance was $p<0.05$. Univariate and multivariate
129 Cox proportional hazard analyses were performed to determine prognostic indicators of
130 OS. Prognostic factors with statistical significance in the univariate analysis were selected
131 for inclusion in the multivariate model. All statistical analyses were performed with EZR
132 [22] (version 1.27, Saitama Medical Center, Jichi Medical University), a graphical user
133 interface for R (R Foundation for Statistical Computing, Vienna, Austria).
134
135 **Results**
136 **Patient characteristics**
137 Patient characteristics are shown in **Table 1**. Sixty-three patients were classified as the
138 mini-MTX group and 20 as the short-MTX group. The median age in the mini-MTX and
139 the short-MTX group were 47 years (range: 16–67) and 48 years (range: 21–67),
140 respectively. The median follow-up period in the mini-MTX group was significantly
141 longer than that in the short-MTX group (1860 days in the mini-MTX group vs. 406 days
142 in the short-MTX group, $p<0.01$). The primary disease was mainly acute leukemia in both
143 of the groups. Although there was no difference statistically, patients with high-risk
144 diseases occurred more frequently in the mini-MTX group than in the short-MTX group.

145 HLA antibody was detected in 11.1% of patients in the mini-MTX group and 5.0% of
146 patients in the short-MTX group, while 33.3% of patients in the mini-MTX group were
147 not assessed for HLA antibody. Among patients who were assessed for HLA antibody, no
148 donor-specific HLA antibody was observed.
149
150 **Engraftment**
151 The median time to neutrophil engraftment did not vary according to the dosage of MTX
152 (20 days, range: 14–37 days in the mini-MTX group vs. 20 days, range: 13–33 days in
153 the short-MTX group, p=0.38). The cumulative incidence of neutrophil engraftment at
154 day 60 was 80.3% (95% confidence interval (CI) 67.7–88.4) in the mini-MTX group and
155 95.0% (95% CI 52.3–99.6) in the short-MTX group (p=0.28) (**Figure 1A**). With respect
156 to the intensity of the conditioning regimens, there was no difference in the cumulative
157 incidence of neutrophil engraftment at day 60 based on the dosage of MTX (for the MAC
158 regimen group: 87.5% (95% CI 71.7–94.9) for the mini-MTX group and 93.8% (95% CI
159 44.0–99.5) for the short-MTX group, p=0.70 and RIC regimen group: 73.1% (95% CI
160 41.4–83.0) for the mini-MTX group and 100% (95% CI 100–100) for the short-MTX
161 group, p=0.47). The median time to platelet engraftment was similar in both the mini-
162 MTX and short-MTX group (53 days, range: 48–60 days and 48 days, range: 39–65 days,
163 p=0.68). The cumulative incidence of platelet engraftment at day 60 tended to be higher
164 in the short-MTX group compared with the mini-MTX group (65.2% (95% CI 50.8–76.3)
165 for the mini-MTX group and 76.7% (95% CI 48.0–90.8) for the short-MTX group,
166 p=0.06) (**Figure 1B**).
167 Graft failure was observed in 6 patients (9.8%) in the mini-MTX group and one patient
168 (5.0%) in the short-MTX group (p=0.68). Among 7 patients with graft failure, HLA

169 antibody was not assessed in only two patinets in the mini-MTX group. The remaining 5
170 patinets were negative for HLA antibody. Four patients received MAC (CY/TBI: 3,
171 Bu/CY: 1), and three received RIC (Flu/L-PAM/TBI: 2, Flu/L-PAM/Bu: 1). Of the 6
172 patients with graft failure in the mini-MTX group, 5 underwent a second allo-HSCT.
173
174 **PIR and ES**
175 The cumulative incidence of PIR in the mini-MTX group and the short-MTX group was
176 59.9% (95% CI: 45.6–71.6) and 69.0% (95% CI: 40.8–85.8) (p=0.85) and the median
177 time to the onset of PIR was 8 days (range: 3–18) and 11 days (range: 6–16) after CBT
178 (p=0.06), respectively. The cumulative incidence of ES in the mini-MTX group and the
179 short-MTX group was 30.8% (95% CI: 14.9–48.3) and 35.5% (95% CI: 5.1–69.7)
180 (p=0.44) and the median time to the onset of ES was 14 days (range: 11–18) and 16 days
181 (range: 9–25) after CBT (p=0.48), respectively. The symptoms of PIR and ES in the mini-
182 MTX group and the short-MTX group were fever (73.5% and 61.5%), skin eruption
183 (79.6% and 100.0%), and jaundice (22.4% and 7.7%). Diarrhea was not observed in
184 patients with PIR and ES in this study. Systemic steroid as the treatment for PIR or ES
185 was administrated in 6.3% of patients in the mini-MTX group and 0.0% of patients in the
186 short-MTX group (p=0.49).
187
188 **Acute GVHD**
189 The cumulative incidence of grade 2–4 and grade 3–4 acute GVHD at day 100 between
190 the mini-MTX group and the short-MTX group was 47.8% (95% CI 32.7–61.5) vs. 57.9%
191 (95% CI 32.1–76.9) (p=0.67) (**Figure 2A**) and 14.3% (95% CI 6.2–25.6) vs. 5.3% (95%
192 CI 0.3–22.0) (p=0.24) (**Figure 2B**), respectively. Grade 4 acute GVHD was observed in

two patients in the mini-MTX group. In detail, one of the two patients occurred grade 4 acute GVHD with skin stage 4 and gut stage 1 but did not receive acute GVHD treatment, and that patient died at day 45 due to the primary disease. The other patient who occurred grade 4 acute GVHD with liver stage 4 and was unresponsive to 1.0 mg/kg of methylprednisolone, died at day 54 due to the progression of acute GVHD. Systemic steroid as the treatment for acute GVHD was administrated in 55.3% of patients in the mini-MTX group and 46.2% of patients in the short-MTX group (p=0.75).

In the subgroup analysis based on disease risk, the cumulative incidence of grade 2–4 acute GVHD at day 100 for the two groups was 50.0% (95% CI 27.4–69.0) for the mini-MTX group and 64.3% (95% CI 32.1–84.2) for the short-MTX group (p=0.59) in patients with a standard-risk disease, and 45.8% (95% CI 25.0–64.5) for the mini-MTX group and 40.0% (95% CI 3.0–78.6) for the short-MTX group (p=0.72) in patients with high-risk disease. In patients with standard-risk disease, the cumulative incidence of grade 3–4 acute GVHD at day 100 in the mini-MTX group tended to be higher rather than that in the short-MTX group (20.0% (95% CI 7.1–37.6) vs. 0.0% (95% CI 0.0–0.0), p=0.08), whereas there was no difference in patients with high-risk disease between the two groups according to the MTX dosage (8.3% (95% CI 1.4–23.7) for the mini-MTX group vs. 20% (95% CI 0.4–62.1) for the short-MTX group, p=0.61).

Chronic GVHD

Chronic GVHD developed in 47.5% (95% CI 24.9–55.0) of the patients from the mini-MTX group and 68.4% (95% CI 42.7–92.6) from the short-MTX group (p=0.17). In comparing patients with chronic GVHD in the mini-MTX and short-MTX group, limited chronic GVHD was observed in 68.2% and 61.5% (p=1.0) of these patients, respectively,

and extensive chronic GVHD in 36.8% and 38.5% (p=1.0), respectively.

NRM

The cumulative incidence of NRM at day 100 and 1 year in the mini-MTX and short-MTX group was 22.2% (95% CI 12.9–33.2) vs. 5.0% (95% CI 0.3–21.1) and 32.0% (95% CI 20.8–43.8) vs. 5.0% (95% CI 0.3–21.1), respectively (p=0.02) (**Figure 3A**). The causes of NRM were as follows: infection (n=7), primary graft failure (n=3), acute GVHD (n=3), hemophagocytic syndrome (HPS) (n=3), sinusoidal obstruction syndrome (SOS) (n=2), secondary malignancy (n=2), and thrombotic microangiopathy (TMA) (n=2) in the mini-MTX group and primary graft failure (n=1) in the short-MTX group (**Table 2**). In the mini-MTX group, 45.5% of NRM occurred before neutrophil engraftment, and half of the deaths were caused by infection. In contrast, 25% of NRM after neutrophil engraftment in the mini-MTX group was due to acute GVHD, and another 25% of that due to HPS. We analyzed the details of complications with a possibility of association with NRM. Regarding the details of infections, there were no significant differences in the incidence of CMV antigenemia and CMV diseases(s) between the mini-MTX and short-MTX group (57.1% vs. 65.0%, p=0.36 and 12.7% vs. 10.0%, p=1.0, respectively). Reactivation of HHV-6 was observed in 4.8% of patients in the mini-MTX group and 0.0% of patients in the short-MTX group (p=1.0). Among patients with HPS, one patient developed HPS at neutrophil engraftment and died on day 40 while the other 2 patients developed HPS due to a primary disease or infection after neutrophil engraftment. The incidence of SOS was 7.9% in the patients in the mini-MTX group and 5.0% in the patients in the short-MTX group (p=1.0). TMA was observed in 14.3% of patients in the mini-MTX group and 10.0% of patients in the short-MTX group (p=1.0).

241 In comparison with the disease risk groups, the cumulative incidence of NRM at 1 year
242 in the mini-MTX group was significantly higher rather than that in the short-MTX group
243 (34.0% (95% CI 17.5–51.2) vs. 0.0% (95% CI 0.0–0.0), p=0.02) in patients in the
244 standard-risk group, although in those in the high-risk group, the incidence did not vary
245 between the two groups (30.3% (95% CI 15.6–46.5) vs. 16.7% (95% CI 0.5–54.9),
246 p=0.54).
247
248 **Relapse and PD**
249 The cumulative incidence of relapse or PD at 1 year was 31.0% (95% CI 19.2–43.6) for
250 the mini-MTX group and 24.2% (95% CI 6.8–47.2) for the short-MTX group (p=0.38)
251 (**Figure 3B**). PFS rate at 1 year in the mini-MTX group was significantly lower compared
252 with that in the short-MTX group (40.4% (95% CI 28.2–52.3) vs. 66.1% (95% CI 39.1–
253 83.3), p=0.03). Among patients with high-risk disease, the cumulative incidence of
254 relapse or PD at 1 year in the mini-MTX group was significantly higher compared with
255 that in the short-MTX group (50.1% (95% CI 30.7–66.8) vs. 0.0%, p=0.04). In contrast,
256 the cumulative incidence of relapse or PD in patients in the standard-risk group at 1 year
257 in the mini-MTX group was lower compared with the short-MTX group (8.7% (95% CI
258 1.4–24.6) vs. 35.7% (95% CI 9.3–63.9), p=0.08).
259
260 **OS**
261 The OS rates at 100 days and 1 year in the mini-MTX group were significantly lower
262 than those in the short-MTX group (68.1% (95% CI 55.1–78.1) vs. 95.0% (95% CI 69.5–
263 99.3) and 46.9% (95% CI 34.1–58.6) vs. 88.7% (95% CI 61.4–97.1), p<0.01, respectively,
264 **Figure 3C**). The OS rates according to the disease risk group are shown in **Figure 4**.

Based on the MTX dosage in patients in the standard-risk disease group, there was no statistically significant difference in the OS rate at 1 year between the mini-MTX group and the short-MTX group (62.4% (95% CI 42.3–77.2) vs. 90.9% (95% CI 50.8–98.7), p=0.16, **Figure 4A**), while in patients in the high-risk group, the OS rate at 1 year in the mini-MTX group tended to be worse compared with those in the short-MTX group (33.3% (95% CI 18.2–49.3) vs. 83.3% (95% CI 27.3–97.5), p=0.07, **Figure 4B**).

Predictive factors for OS

We performed univariate analysis to determine predictive factors for OS (**Table 3**). Among all prognostic factors with statistical significance in univariate analysis, the year of transplantation was divided into 2 groups (i.e., 2006-2012 and 2013-2018) because the median transplant date in 83 patients was in September 2012. In multivariate analysis, dosage of MTX (mini-MTX) and high-risk disease were the independent prognostic factors for OS (hazard ratio (HR): 4.11; 95% CI: 1.15–14.65; p=0.03 and HR: 1.93; 95% CI: 1.03–3.60; p=0.04, respectively, **Table 3**).

Discussion

We demonstrated that mini-MTX for GVHD prophylaxis was the most powerful predictive factor for OS in multivariate analysis, which was caused by higher NRM rate in the mini-MTX group. Neutrophil engraftment was not associated with the dosage of MTX in GVHD prophylaxis.

Recently, several studies that focused on the dosage of MTX combined with TAC for GVHD prophylaxis in CBT were reported (**Table 4**) [12, 13] [23, 24]. In this study, there was no difference in the median time and cumulative incidence of neutrophil engraftment

289 between the mini-MTX and the short-MTX group. The median time to neutrophil
290 engraftment was reported to be 20.5 days in the study by Fuji S et al. [24], which used
291 the same GVHD prophylactic regimen as the mini-MTX group in our study, and 21 days
292 in the study by Adachi Y et al. [23], which evaluated the same GVHD regimen as the
293 short-MTX group in our study. These results were consistent with our study. Considering
294 these results, there is no association between the dosage of MTX for GVHD prophylaxis
295 in CBT and the median time for neutrophil engraftment. In contrast, the cumulative
296 incidence of neutrophil engraftment in the previous study that used the mini-MTX
297 prophylaxis regimen [24] was higher than that in the mini-MTX group of our study
298 (90.0% vs. 80.3%). NRM in our mini-MTX group occurred in 10 patients before
299 neutrophil engraftment. Among these patients, 5 patients died due to infections and 3 to
300 primary graft failure. A possible reason may be that the management strategies for
301 infection or the screening for the HLA antibody were not established because all the
302 patients in our mini-MTX group underwent CBT between 2006 and 2016.
303 The incidence of grade 2–4 and 3–4 acute GVHD at day100 in this study was more
304 frequent than their counterparts in the previous study that used the mini-MTX prophylaxis
305 regimen [24] (47.8% vs. 35.0% for grade 2–4 acute GVHD and 14.3% vs. 5.0% for grade
306 3–4 acute GVHD). Several risk factors for acute GVHD have been reported.
307 Hematopoietic stem cell source, hematopoietic transplantation-comorbidity index (HCT-
308 CI), intensity of conditioning regimen, disease risk at transplantation, and T-cell depletion
309 can influence the development of acute GVHD [25-28] [29, 30]. The differences in patient
310 characteristics, such as disease risk, conditioning regimen, or year of transplantation, may
311 be the reason for the differences in the incidence of acute GVHD between the two studies.
312 Conversely, although these were not statistically significant, the incidence of grade 3–4

313 acute GVHD in the short-MTX group was lower than that in the mini-MTX group. Adachi
314 Y, et al. also demonstrated that a higher incidence of grade 3–4 acute GVHD in patients
315 using MTX at dose of 10-7-7 mg/m^2 was observed compared with that in MTX dose of
316 15-10-10 mg/m^2 (24.3% *vs.* 3.0%, p=0.01) [23]. These results indicate that an adequate
317 dosage of MTX for GVHD prophylaxis is important to prevent the incidence of severe
318 acute GVHD even in CBT.

319 The most important finding of this analysis is that because of higher NRM in the mini-
320 MTX group, the OS in this group was significantly lower than that in the short-MTX
321 group. The causes of NRM were mainly infections, primary graft failure before neutrophil
322 engraftment, and acute GVHD after neutrophil engraftment. In contrast, the study by Fuji
323 et al evaluated mini-MTX GVHD prophylaxis and showed the lower incidence of NRM
324 and the higher OS rate [24] compared with that in our study (5.0% vs. 32.0% for NRM at
325 1 year and 85.0% vs. 46.9% for the OS rate at 1 year). The mentioned above, the higher
326 NRM due to infections before engraftment and acute GVHD after engraftment in our
327 study may be explained by the difference in implementation or year of transplantation.
328 Adachi Y et al reported that transplant-related complications with endothelial cell damage,
329 including TMA, were more frequently observed in patients with GVHD prophylaxis with
330 MTX at the dose of 10-7-7 mg/m^2 compared with MTX at the dose of 15-10-10 mg/m^2
331 [23]. In our study, the incidence of TMA was not different in the mini-MTX and short-
332 MTX group. Although we also considered the other transplant-related complications
333 including SOS and CMV and HHV-6 infections, there was no significant difference in the
334 incidence of these factors.

335 MTX combined with CNI for GVHD prophylaxis in CBT is more frequently used in
336 Japan, whereas mycophenolate mofetil (MMF) is frequently used in combination with

337 CNI in European countries and the United States [31]. The incidence of severe acute
338 GVHD in patients with MTX combined with CNI was significantly lower than that in
339 those with MMF combined with CNI [32, 33]. The most recent report in AML patients
340 who underwent CBT [34] showed that the incidence of grade 2–4 acute GVHD in the
341 mini-MTX or MMF combined with TAC was significantly higher than that in MTX dose
342 of 15-10-10 mg/m^2 combined with TAC. On the other hand, the incidence of grade 3–4
343 acute GVHD was not different in each dose group of the MTX and MMF group combined
344 with TAC or cyclosporine A. In addition, there was no difference in the incidence of
345 neutrophil recovery and OS among the GVHD prophylaxis groups, including various
346 MTX dosages and MMF, despite the potential background differences. It will be
347 important to reevaluate which MTX or MMF combined with CNI for GVHD prophylaxis
348 affects clinical outcomes including OS in CBT in matched background characteristics.
349 There are some limitations of this study. This study is retrospective and it compares a
350 historical cohort, and there are also many differences in the clinical features, conditioning
351 regimens or disease risk, and the management for complication between the mini-MTX
352 and the short-MTX group. Additionally, the sample size was relatively small, and the
353 follow-up period was short in the short-MTX cohort. This potential for bias may also have
354 had an effect on the accuracy of the analysis.
355 However, despite these limitations, we demonstrated that the dosage of MTX combined
356 with TAC regimen for GVHD prophylaxis was the most important predictive factor for
357 successful outcome in patients who underwent CBT. Although the reduced dosage of
358 MTX for GVHD prophylaxis had no effect on neutrophil engraftment, the increased NRM
359 due to higher incidence of infection, graft failure, and severe acute GVHD was associated
360 with a lower survival rate in the mini-MTX group.

Although our results can provide guidance in clinical decisions for GVHD prophylaxis in CBT, future studies on a larger scale are needed in order to determine the optimal dosage of MTX for GVHD prophylaxis in CBT.

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Conflict of interest

The authors declare that they have no conflict of interest.

Reference

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Figure legends

Figure 1.

Cumulative incidence of neutrophil and platelet engraftment. (A) Cumulative incidence of neutrophil engraftment and (B) cumulative incidence of platelet engraftment in the mini-MTX and short-MTX group.

Figure 2.

Cumulative incidence of acute GVHD. (A) Cumulative incidence of grade 2–4 acute GVHD and (B) cumulative incidence of grade 3–4 acute GVHD in the mini-MTX and short-MTX group.

Figure 3.

Cumulative incidence of non-relapse mortality and the relapse or progression disease and the probability of overall survival. (A) Cumulative incidence of non-relapse mortality, (B) cumulative incidence of the relapse or progression disease, and (C) overall survival in the mini-MTX and short-MTX group.

Figure 4.

Probability of overall survival in the mini-MTX group and the short-MTX group, based on disease risk. Kaplan-Meier curves are shown for each category of disease risk at transplantation. (A) Standard-risk disease and (B) high-risk disease.

Figure 1.

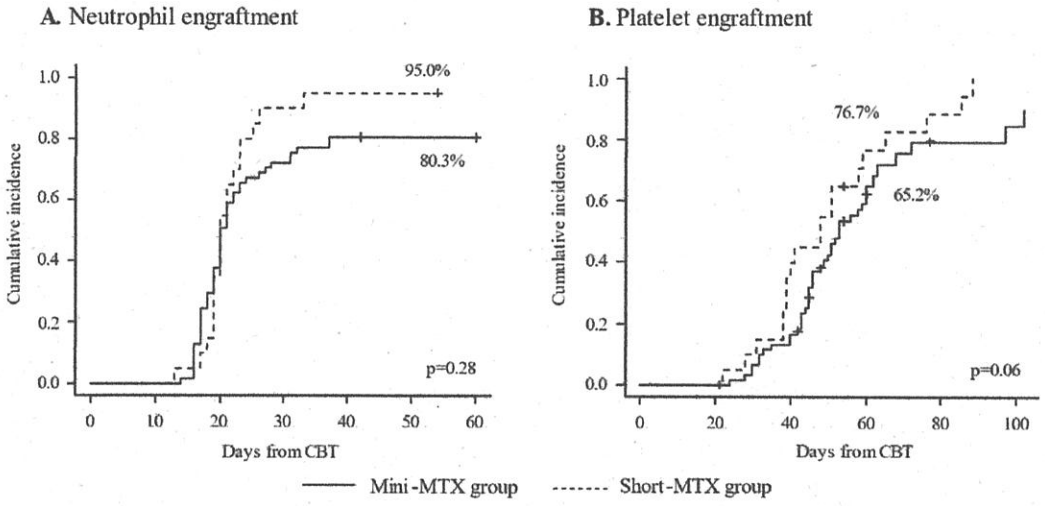

Figure 1. Cumulative incidence of neutrophil and platelet engraftment based on dosage of MTX. (A) Cumulative incidence of neutrophil engraftment and (B) cumulative incidence of platelet engraftment in the mini-MTX and short-MTX group.

Figure 2.

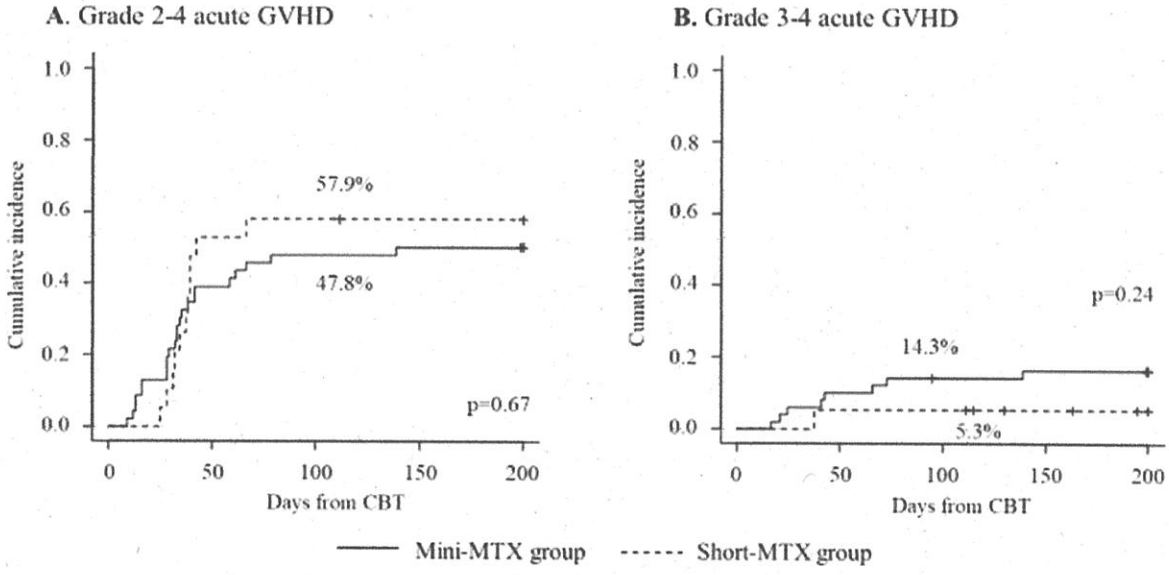

Figure 2. Cumulative incidence of acute GVHD based on dosage of MTX. (A) Cumulative incidence of grade 2–4 acute GVHD and (B) cumulative incidence of grade 3–4 acute GVHD in the mini-MTX and short-MTX group.

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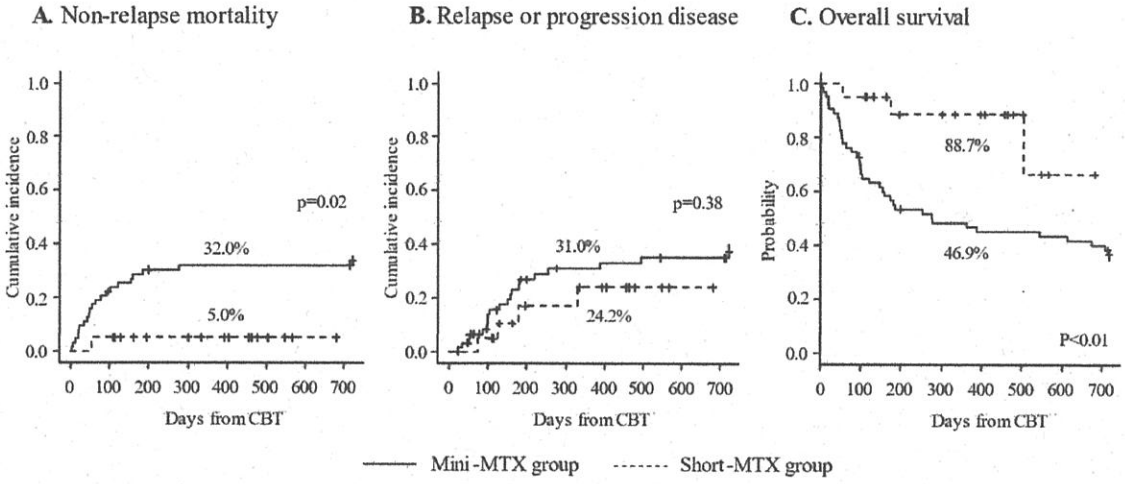

Figure 3. Cumulative incidence of non-relapse mortality and the relapse or progression disease and the probability of overall survival based on dosage of MTX. (A) Cumulative incidence of non-relapsed mortality, (B) cumulative incidence of the relapse or progression disease, and (C) overall survival in the mini-MTX and short-MTX group.

Figure 4.

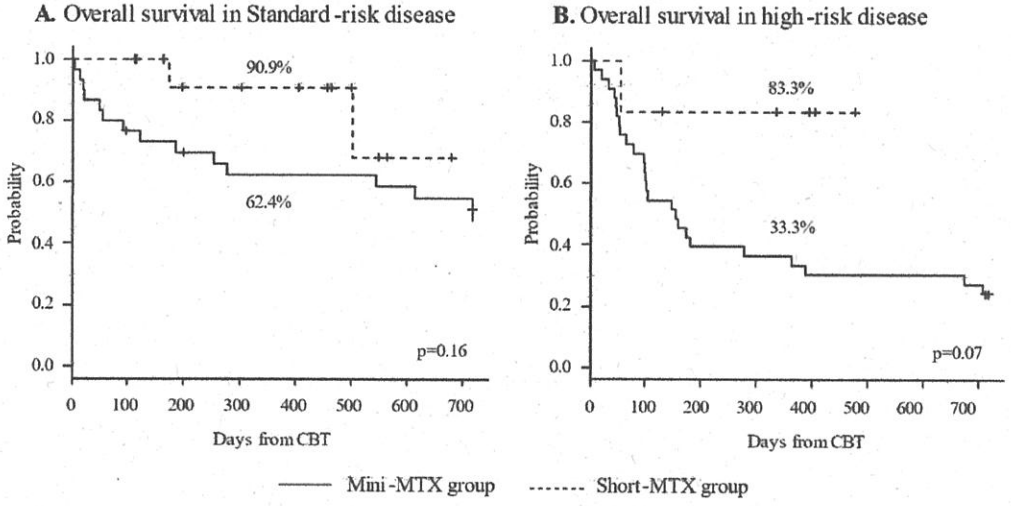

Figure 4. Probability of overall survival compared with disease risk in the mini-MTX group and the short-MTX group, based on disease risk. Kaplan-Meier curves are shown for each category of disease risk at transplantation. (A) Standard-risk disease and (B) high-risk disease.

Table 1 Patient characteristics

Clinical featurers	Mini-MTX (n=63)	Short-MTX (n=20)	*P* value
Age, median (range)	47 (16-67)	48 (21-67)	0.64
Primary disease			
AML	26 (41.3%)	7 (35.0%)	0.57
ALL	13 (20.6%)	6 (30.0%)	
MDS	10 (15.9%)	5 (25.0%)	
ML	11 (17.5%)	1 (5.0%)	
CML	1 (1.6%)	0 (0.0%)	
MPD	1 (1.6%)	0 (0.0%)	
PMF	1 (1.6%)	0 (0.0%)	
CAEBV	0 (0.0%)	1 (5.0%)	
Disease risk			
Standard	30 (47.6%)	14 (70.0%)	0.12
High	33 (52.4%)	6 (30.0%)	
HCT-CI			
0	39 (61.9%)	12 (60.0%)	0.51
1	17 (27.0%)	4 (20.0%)	
2	4 (6.3%)	1 (5.0%)	
3	2 (3.2%)	2 (10.0%)	
4	1 (1.6%)	1 (5.0%)	
Conditioning regimen			
MAC	42 (66.7%)	16 (80.0%)	0.4
CY+TBI based	26 (62.0%)	9 (56.2%)	
L-PAM+TBI based	10 (23.9%)	1 (6.3%)	
Bu+CY	2 (4.7%)	0 (0.0%)	
Flu+L-PAM+Bu	2 (4.7%)	6 (37.5%)	
Flu+Bu+TBI	2 (4.7%)	0 (0.0%)	
RIC	21 (33.3%)	4 (20.0%)	
Flu+L-PAM	2 (9.5%)	0 (0.0%)	
Flu+L-PAM+TBI	18 (85.7%)	3 (75.0%)	
Flu+L-PAM+Bu (≤6.4mg/kg)	1 (4.8%)	1 (25.0%)	
TNCC number (10^7/kg)			
Median (range)	2.87 (1.85-6.25)	2.99 (1.98-4.07)	0.31
Number of antigen level mismatches			
0	9 (14.3%)	1 (5.0%)	0.37
1	15 (23.8%)	3 (15.0%)	
2	39 (61.9%)	16 (80.0%)	
HLA antibody			
Negative	35 (55.6%)	19 (95.0%)	<0.01
Positive	7 (11.1%)	1 (5.0%)	
Not assessed	21 (33.3%)	0 (0.0%)	
Leucovorin rescue			
Performed	42 (66.7%)	8 (40.0%)	0.04
Not performed	21 (33.3%)	12 (60.0%)	
TAC administration period (days)			
Median (range)	118 (2-2698)	191 (30-650)	0.24

AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, MDS: myelodysplastic syndrome, ML: malignant lymphoma, CML: chronic myeloid leukemia, MPD: myeloproliferative disorders, PMF: primary myelofibrosis, CAEBV: chronic active Epstein-Barr virus, HCT-CI: hematopoietic cell transplantation-comorbidity index, MAC: myeloablative conditioning, RIC: reduced intensity conditioning, CY: cyclophosphamide, TBI: total body irradiation, L-PAM: melphalan, Bu: busulfan, Flu: fludarabine, TNCC: total nucleated cell count, TAC: tacrolimus

Table 2 Causes of non-relapse mortality in the mini-MTX and short-MTX group

Factors	Mini-MTX n=22		Short-MTX n=1	
Before or after neutrophil engraftment	Before n=10	After n=12	Before n=1	After n=0
Infection	5 (50%)	2 (16.7%)	0 (0%)	0 (0%)
Graft failure	3 (30%)	N/A	1 (100%)	N/A
Acute GVHD	N/A	3 (25%)	N/A	0 (0%)
HPS	0 (0%)	3 (25%)	0 (0%)	0 (0%)
SOS	1 (10%)	1 (8.3%)	0 (0%)	0 (0%)
Secondary malignancy	0 (0%)	2 (16.7%)	0 (0%)	0 (0%)
TMA	1 (10%)	1 (8.3%)	0 (0%)	0 (0%)

MTX: methotrexate, SOS: sinusoidal obstruction syndrome, HPS: hemophagocytic syndrome, TMA: thrombotic microangiopathy, N/A: not applicable

Table 3 Prognostic factors affecting overall survival

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (>50)	1.10 (0.60-2.01)	0.75		
PS (≥2)	1.51 (0.64-3.59)	0.35		
Disease risk (High risk)	2.18 (1.18-4.03)	0.01	1.93 (1.03-3.60)	0.04
HCT-CI (≥2)	0.49 (0.15-1.57)	0.23		
CMV status (antibody positive)	1.38 (0.61-3.13)	0.44		
Conditionig regimen (RIC)	1.14 (0.60-2.16)	0.69		
Transplant years (2006-2012)	1.70 (1.90-3.21)	0.10	0.92 (0.46-1.83)	0.81
Dosage of MTX (mini-MTX)	4.44 (1.36-14.45)	0.01	4.11 (1.15-14.65)	0.03
Leucovorin rescure (not performed)	1.62 (0.84-3.11)	0.15		

PS: performance status, HCT-CI: hematopoietic cell transplantation-comorbidity index, RIC; reduced intensity conditioning, MTX: methotrexate, HR: hazard ratio, CI: confidence interval

Table 4 Outcomes of cord blood transplantation using the combination of tacrolimus and methotrexate for GVHD prophylaxis

	MTX (mg/m^2) (day 1-3-6)	Patients (n)	Age (Median)	High-risk disease (%)	MAC (%)	Neutrophil engraftment		Acute GVHD		NRM (%)	Relapse or PD (%)	OS (%)
						Rate (%)	Median time (days)	Grade2-4 (%)	Grade3-4 (%)			
Lekakis et al.	5-5-5[+]	15	34	NR	NR	NR	22	22.2	0	53.0 (1 year)	NR	13.0 (4 years)
Fuji et al.	5-5-5	20	54.5	40.0	15.0	90.0	20.5	35.0	5.0	5.0 (1 year)	15.0 (1 year)	85.0 (1 year)
Saito et al.	10-7	40	60.5	67.5	70.0	92.5	21	48.1	NR	25.3 (1 year)	25.2[++] (1 year)	58.6 (1 year)
Adachi et al.	10-7-7	37	55	45.9	73.0	97.3	21	43.2	24.3*	16.3 (2 years)	27.0[++] (2 years)	56.6 (2 years)
	15-10-10	33	43	33.3	84.8	93.9	21	41.2	3.0*	6.1 (2 years)	15.3[++] (2 years)	84.8 (2 years)
Present study	5-5-5	63	47	52.4	66.7	80.3	20	47.8	14.3	32.0** (1 year)	31.0 (1 year)	46.9*** (1 year)
	10-7-7	20	48	30.0	80.0	95.0	20	57.9	5.3	5.0** (1 year)	24.2 (1 year)	88.7 *** (1 year)

+ : all patients received rabbit anti-thymocyte globulin (ATG) as GVHD prophylaxis, ++ : calculated as cumulative incidence of relapse, * : p-value=0.01, **: p-value=0.02, ***: p-value <0.01, MTX: methotrexate, MAC: myeloablative conditioning, NRM: non-relapse mortality, PD: Progression disease, OS: Overall survival, NR: not reported