TITLE

Bone marrow graft-versus-host disease: evaluation of its clinical impact on disrupted hematopoiesis after allogeneic hematopoietic stem cell transplantation

AUTHOR & AFFILIATIONS

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Key words:

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ABSTRACT

Idiopathic cytopenias are frequently observed in patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). We have previously reported the effect of graft-versus-host disease (GVHD) on bone marrow (BM) in murine models, indicating that the osteoblast injury mediated by donor T cells was associated with bone marrow suppression and delayed immune reconstitution (“BM GVHD”). In this study, we prospectively evaluated the relevance of these findings in 51 patients. Patients with chronic GVHD manifested the loss of osteoblasts, contributing to cytopenic symptoms ($P = 0.0427$, vs. patients without cytopenic symptoms). The loss of osteoblasts was significantly associated with the extensive type of chronic GVHD ($P = 0.012$) and flow cytometric analyses revealed lower numbers of $\text{CD}19^+$ B cells and significantly increased $\text{CD}4/\text{CD}8$ ratio ($P = 0.0002$) in these patients. Our data for the first time summarize the detailed analyses of the effect of GVHD on BM in the clinical allo-HSCT patients.
INTRODUCTION

Allogeneic stem cell transplantation (allo-HSCT) is currently established as a curable therapy for hematologic malignancies. However, graft-versus-host disease (GVHD) still remains as a major complication after allo-HSCT and therefore development of better strategies for the prophylaxis and treatment of GVHD are essential to improve outcomes of allo-HSCT. The principal target organs of acute GVHD are the skin, liver, gastrointestinal tract (1). However, cytopenias and bone marrow (BM) suppression are often observed in association with GVHD in patients undergoing allo-HSCT, suggesting that BM is a potential target of GVHD. Clinical and experimental data have shown that immunologic reconstitution is impaired by GVHD (2-5), and GVHD-associated myelosuppression and lymphoid hypoplasia have been reported (6-8). Recently, we demonstrated the destruction of BM hematopoietic niches, especially osteoblasts by donor T cells in murine models of GVHD, resulting in BM suppression, including B-lymphopoiesis. We identified this phenomenon as “BM GVHD” (9). Here we report a clinical research for the investigation of BM GVHD in patients after allo-HSCT. We analyzed 51 patients undergoing allo-HSCT who were evaluable with BM biopsy samples both before and after allo-HSCT.
METHODS

Study Design and Patients

We enrolled 57 patients for our prospective analyses of BM GVHD who underwent allo-HSCT from February, 2010 to June, 2012 in Hokkaido University Hospital. A total of 51 patients were assessed for BM biopsy specimens before and after allo-HSCT (6 patients who did not experience BM biopsies at all after allo-HSCT were excluded). The study protocol was approved by the review board of Hokkaido University Graduate School of Medicine on January 29th, 2010. Patients provided written informed consent before being enrolled in the protocol. Characteristics of patients as well as transplantation procedures are summarized in Table 1.

Evaluation of GVHD

Diagnosis and clinical grading of acute and chronic GVHD were performed according to the established criteria (10-12).

Bone marrow samples

We performed BM biopsies and aspirations for patients before and after allo-HSCT. BM aspirates were analyzed for B and T-cell profiles by flow cytometry. Biopsied specimens were stained with hematoxylin and eosin, as well as with CD56 for immunohistochemical assessments of cellularity, morphology and presence/absence of osteoblasts (13). We categorized the loss of osteoblasts into three groups. 'Not affected' if the osteoblasts are intact or decrease in number is moderate up to 30%, 'Partial loss' if the osteoblasts are partially lost in between 30% to 90% of the bone trabeculae in the pathological sections, and 'Complete loss' if more than 90% of osteoblasts are lost.

Assessment of cytopenias
We determined the cytopenic condition as *idiopathic cytopenias* after excluding following conditions. We excluded bacterial, fungal and viral infections by routine screening tests (serological as well as culture tests). Additionally, thrombo microangiopathy (TMA), and hemophagocytic syndrome (HPS) that also cause cytopenias in patients are excluded. Insufficient hematopoiesis after engraftment was also excluded when the patient showed recovery (confirmed retrospectively) in hematopoiesis without any specific treatment for the cytopenias.

**Statistical Analysis**

Median values and ranges were used for continuous variables and percentages for categorical variables (Table 1). Gray’s test was used for group comparisons of cumulative incidences of acute and chronic GVHD. Statistical analyses were performed using Chi-square test and *t*-test, as appropriate. JMP software version 8.0.2 (SAS Institute, Cary, NC, USA) was used for most of the statistical analyses. Analysis of cumulative incidences was carried out with EZR (Saitama Medical Center, Jichi Medical University, http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0) (14). All *P*-values were two sided, and a value of *P* = 0.05 was used as a cut off for statistical significance.
RESULTS

Patients’ characteristics, samples collections and acute and chronic GVHD after allo-HSCT

The patients’ characteristics are shown in Table 1. The median age at allo-HSCT for the 27 males and 24 females was 41 years (range, 19 – 66). Of 51 patients analyzed, 32 developed acute GVHD (Figure 1A), and 29 developed chronic GVHD (Figure 1B). BM biopsies were performed before (median day -22; range, day -174 to day -8), and after (median day 63; range, day 18 to day 527) allo-HSCT together with BM aspirations. The average number of the BM biopsy performed after allo-HSCT was 1.7 times per patient (range, 1 to 6 times). We found a significant decrease of BM cellularity in samples collected from patients suffering from cytopenias in the peripheral blood (Supplemental Table 1). The characteristics of GVHD in terms of the duration from its onset to BM biopsy as well as the percentage of donor chimerism in the samples with cytopenic symptoms are shown in Supplemental Table 2.

BM GVHD and BM suppression during acute GVHD

We analyzed a total of 56 BM samples biopsied from day 0 to day 100 after allo-HSCT (Figure 2). Of these 56 samples, 15 were harvested when the patients had acute GVHD. Eight samples out of these 15 were harvested from the patients suffering from cytopenias and 2 of them displayed partial loss of osteoblasts, identifying 1 sample as idiopathic cytopenias. We also identified 3 samples presenting the partial loss of osteoblasts, however none of these 3 samples were collected when patients showed clinical manifestation of acute GVHD symptoms. The causes of cytopenias for these 3 samples included disease relapse, and delayed engraftment. Taken together, during
early period after allo-HSCT, we did not observe strong correlation between loss of osteoblasts and idiopathic cytopenias.

**BM GVHD and BM suppression during chronic GVHD**

We analyzed a total of 33 samples biopsied beyond day 100 (Figure 3A). Of 14 samples harvested from patients when they exhibited symptoms of chronic GVHD and concurrent idiopathic cytopenias, 4 samples displayed partial loss of osteoblasts and another 6 samples displayed complete loss of osteoblasts. We further detailed the types of chronic GVHD affecting these idiopathic cytopenias and found 10 samples out of 19 samples suffering from idiopathic cytopenias displayed extensive chronic GVHD (Figure 3B). The loss of osteoblasts was significantly correlated with the extensive type of chronic GVHD (Table 2, \( P = 0.012 \)) and also with idiopathic cytopenias in patients with chronic GVHD (Table 3, \( P = 0.0427 \)). Of samples collected when patients had no cytopenic symptoms, no loss of osteoblast was observed. We observed significantly higher frequency of GVHD treatment with steroids in patients with osteoblast loss during chronic GVHD (Table 4). Characteristic pathological analyses of these cases as well as a control BM sample are summarized below.

**Case 1: A patient with no GVHD and cytopenias**

Figure 4A indicates a pathological sample from a 47-year-old female who had no episodes of GVHD symptoms and cytopenias when her BM sample was harvested on day 41. In hematoxylin and eosin staining osteoblasts lining bone trabeculae are well observed before allo-HSCT and on day 41 (arrow heads). The lower panels show CD56 staining from the same patient. Previous reports indicates neural cell adhesion molecule (NCAM, CD56) is strongly expressed by human osteoblasts (13), therefore we utilized CD56 staining for our samples to specifically identify osteoblasts.
Case 2: A patient with chronic liver and skin GVHD with cytopenias

A 57-year-old male patient underwent allo-HSCT from a HLA identical sibling. His underlining disease (anaplastic large cell lymphoma) relapsed on day 134 and tacrolimus was tapered off afterwards. Chronic extensive GVHD of the liver manifested on day 168, followed by deterioration of cutaneous and oral chronic GVHD and cytopenias including platelets and red blood cells (grade 4 in platelets and grade 2 in hemoglobin by Common Terminology Criteria for Adverse Events (CTCAE) version 4). BM biopsy on day 167 shows complete loss of osteoblasts (Figure 4B). Chronic GVHD and cytopenias were then improved by the resumption of low-dose tacrolimus and BM biopsy on day 521 showed recovery of osteoblasts.

Case 3: A patient with sustained cytopenias with skin GVHD

Figure 4C shows BM from a 37-year-old male patient receiving allo-HSCT from an HLA mismatched unrelated donor. Gradual cytopenias was observed from day 90 after allo-HSCT with stage 1 cutaneous GVHD. BM biopsy on day 127 displays complete loss of osteoblasts. When he recovered from these symptoms, osteoblasts reappeared on the sample taken on day 260.

Case 4: A patient with gradual loss of osteoblasts with worsening GVHD

A 27-year-old male patient underwent allo-HSCT from an unrelated donor. He achieved neutrophil engraftment on day 20, however he continued to be dependent on platelet and red blood cell transfusions. He developed stage 1 skin GVHD on day 34. The red blood cell and platelet engraftment were achieved on day 106 (reticulocytes > 1%) and day 30 (>20,000/ml), respectively. Osteoblasts were partially lost on BM samples taken on day 36 (Figure 4D). Cytopenias continued and he then developed chronic lung GVHD exacerbating form day 97, which was successfully treated with
steroid therapy. However the cytopenias persisted and the complete loss of osteoblasts was observed in BM samples taken on day 147. These results demonstrate the potential correlation between systemic (and supposedly affecting BM) GVHD and loss of BM osteoblasts leading to cytopenias. The patient did not develop bronchiolitis obliterans syndrome.

**Suppression of CD19\(^+\) B cells and increased CD4/CD8 ratio in BM samples with the loss of osteoblasts**

We next examined the BM aspirates samples taken at the same time points of the BM biopsies. We analyzed samples collected after day 100 by categorizing them into three subgroups based on the status of osteoblasts – not affected (NA), partial loss (PL), and complete loss (CL). We observed decreased numbers of CD19\(^+\) B cells and CD3\(^+\) T cells in parallel with the loss of osteoblasts (Figure 5). Also, we found the ratio of CD4 and CD8 T cells was significantly increased with the loss of osteoblasts \((P = 0.0002, \text{NA vs. CL})\). These data indicate the effects of BM GVHD resulting in disrupted hematopoiesis after allo-HSCT and are consistent with our mouse model data in the setting of BM GVHD (9).
DISCUSSION

In the settings of clinical allo-HSCT, patients frequently suffer from sustained cytopenias that parallels systemic GVHD. Some patients in the outpatient clinics after day 100 even develop cytopenias without any signs of infection or GVHD. The causes for these cytopenias include relapse of original disease, viral (or bacterial) infections, and/or side effects of drugs, and it is very important to identify the cause as it directly affects the decision making for treating these patients. By evaluating BM biopsy samples with hematoxylin and eosin as well as CD56 immunohistochemical staining, we analyzed BM osteoblasts and compared these results with the respective clinical courses. As a result, we confirmed the disappearance of osteoblasts in the samples of patients with idiopathic cytopenias and chronic GVHD during the late stage after allo-HSCT, suggesting the correlation between chronic GVHD and BM GVHD, resulting in BM suppression. Bone damage after allo-HSCT (15), especially suppression of B lymphopoiesis during GVHD has been reported in both clinical and experimental studies (2, 6, 16-19) and our previous study unveiled new details of the mechanisms involved in this phenomenon, focusing on the destruction of hematopoietic niches by donor T cells in the course of GVHD in murine models (9). In this article, we also reported the analyses on various clinical factors in BM; including cellularities and B-cell analyses, which indicated the correlation with BM GVHD in human chronic GVHD cases. Consistent with our findings in murine GVHD models, we observed decreased number of CD19^+ B cells and increased CD4/CD8 ratio in patients with osteoblast destruction, however these findings were not observed during early period after allo-HSCT when in murine models donor CD4^+ T cells mediated strong BM GVHD. It is possible that in the clinical settings patients are treated with immunosuppressive therapy and this could
have contributed to prevent acute BM GVHD (20, 21). In cases of patients under treatment of steroids, it is quite difficult to separate the effects of chronic GVHD on osteoblasts from those of its treatments with steroids, as steroids also decrease osteoblastic proliferation and activity (22). We observed higher frequency of GVHD treatment with steroids in patients who had idiopathic cytopenias with osteoblast loss (Table 4), indicating more severe systemic GVHD with BM GVHD that required steroids therapy.

In conclusion, we have shown for the first time the direct proof of BM GVHD and the loss of osteoblasts in chronic GVHD patients. Further studies with large number of patients are warranted, however our findings explain the cause of idiopathic cytopenias after allo-HSCT and give valuable clues for clinicians to treat patients suffering from BM suppression after allo-HSCT.

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Conflict of interest statement: There are no conflicts of interest to report.
### Table 1. Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. of transplants</td>
<td>51</td>
</tr>
<tr>
<td>Median patient age (range)</td>
<td>41 (19-66)</td>
</tr>
<tr>
<td>Patient sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (53%)</td>
</tr>
<tr>
<td>Female</td>
<td>24 (47%)</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>16 (31%)</td>
</tr>
<tr>
<td>MDS</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>ALL/LBL</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>ML</td>
<td>9 (18%)</td>
</tr>
<tr>
<td>ATL</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>AA</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Others</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Donor sources</td>
<td></td>
</tr>
<tr>
<td>U-BMT</td>
<td>28 (55%)</td>
</tr>
<tr>
<td>R-PBSCT</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>R-BMT</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>CBT</td>
<td>10 (20%)</td>
</tr>
<tr>
<td>Preparative regimen</td>
<td></td>
</tr>
<tr>
<td>CST</td>
<td>20 (39%)</td>
</tr>
<tr>
<td>RIST</td>
<td>31 (61%)</td>
</tr>
<tr>
<td>Immuno suppression</td>
<td></td>
</tr>
<tr>
<td>CyA based</td>
<td>13 (25%)</td>
</tr>
<tr>
<td>FK based</td>
<td>38 (75%)</td>
</tr>
</tbody>
</table>

AML: acute myelogenous leukemia; MDS: myelodysplastic syndrome; ALL/LBL: acute lymphocytic/lymphoblastic lymphoma; ML: malignant lymphoma; ATL: acute T-cell leukemia; AA: aplastic anemia; U-BMT: unrelated bone marrow transplantation; R-PBSCT: related peripheral blood stem cell transplantation; R-BMT: related BMT; CBT: cord blood transplantation; CST: conventional stem cell transplantation; reduced intensity stem cell transplantation; CyA: cyclosporin A; FK: tacrolimus

### Table 2. Loss of osteoblasts and its correlation with chronic GVHD in BM samples after day 100

<table>
<thead>
<tr>
<th>chronic GVHD</th>
<th>Loss of osteoblasts (+)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any chronic GVHD</td>
<td>11 (n = 14 samples)</td>
<td>0.0324</td>
</tr>
<tr>
<td>Limited chronic GVHD</td>
<td>3 (n = 19 samples)</td>
<td>0.7451</td>
</tr>
<tr>
<td>Extensive chronic GVHD</td>
<td>8 (n = 19 samples)</td>
<td>0.0120</td>
</tr>
</tbody>
</table>
Table 3. Loss of osteoblasts and its correlation with idiopathic cytopenias in BM samples after day 100

<table>
<thead>
<tr>
<th>Loss of osteoblasts*</th>
<th>cGVHD(+), idiopathic cytopenia (+) n = 14 samples</th>
<th>cGVHD(+), idiopathic cytopenia (-) n = 5 samples**</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any osteoblast loss</td>
<td>10</td>
<td>1</td>
<td>0.0427</td>
</tr>
<tr>
<td>Partial loss</td>
<td>4</td>
<td>1</td>
<td>0.7032</td>
</tr>
<tr>
<td>Complete loss</td>
<td>6</td>
<td>0</td>
<td>0.0324</td>
</tr>
</tbody>
</table>

*Loss of osteoblasts is defined as partial: partial loss of osteoblasts is observed in 30-90% of the bone trabeculae in the pathological sections; complete: more than 90% of the osteoblast are lost.

**These samples include n = 3 samples without cytopenias and n = 2 with cytopenias with identified causes.

Table 4. Steroid administration and loss of osteoblasts in chronic GVHD BM samples after day 100

<table>
<thead>
<tr>
<th>Steroid therapy at BM biopsy</th>
<th>Loss of osteoblasts (+) cGVHD(+) n = 11 samples</th>
<th>Loss of osteoblasts (-) cGVHD(+) n = 8 samples</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>8</td>
<td>2</td>
<td>0.0360</td>
</tr>
<tr>
<td>(-)</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1.** Cumulative incidences of acute (A) and chronic (B) GVHD after allo-HSCT.

**Figure 2.** Assessments of BM biopsy samples from patients up to day 100 after allo-HSCT. aGVHD: acute GVHD; Bx: biopsy.

**Figure 3.** Assessments of BM biopsy samples from patients after day 100 after allo-HSCT. (A) Relations between loss of osteoblasts and idiopathic cytopenias. (B) Grades of chronic GVHD and their relations to idiopathic cytopenias.

**Figure 4.** Loss of osteoblasts during GVHD and cytopenias. Biopsied BM samples were stained with hematoxylin and eosin (upper panels for all pictures), as well as with CD56 (lower panels for all pictures) for assessments of cellularity, morphology and presence/absence of osteoblasts. Magnification for the images is ×400. (A) BM samples from a 47-year-old patient (post U-BMT) who had no episode of GVHD and cytopenias when BM biopsies were performed. Osteoblasts are well preserved. Arrow heads indicate osteoblasts. (B) A 57-year-old patient who underwent R-PBSCT and relapsed on day 134 developed symptoms of chronic GVHD and cytopenias after day 160. Complete loss of osteoblasts is shown on day 176, and when he recovered from those symptoms osteoblasts are back on day 521. (C) A 37-year-old patient after R-PBSCT gradually developed cytopenias after day 90 with stage 1 skin GVHD. The BM samples on day 127 show clear loss of osteoblasts. On day 260 when his symptoms subsided, osteoblasts were recovered to a normal level. (D) A 27-year-old patient who underwent U-BMT attained engraftment of white blood cells on day 20, however cytopenias had persisted and he developed stage 1 skin GVHD on day 34. Partial loss of osteoblasts was observed from day 36 to day 68 and he had been on high demand of red blood cell
and platelet transfusions. The BM samples on day 147 indicate complete loss of osteoblasts.

**Figure 5.** Flow cytometric analyses of BM aspirates in patients after day 100. BM aspiration was performed at the same time of BM biopsy in these patients. Cellularity, nucleated cell count (NCC), CD19⁺ cells, CD3⁺ cells, and the ratio of CD4⁺ and CD8⁺ cells are shown.
REFERENCES


Figure 1

(A) Cumulative incidence

- Total: 62.7%
- Grade ≥2: 37.5%

(B) Cumulative incidence

- Total: 59.4%
- Extensive: 37.5%

Days after allo-HSCT

Figure 2

BM Bx Samples ~day100

- aGVHD at Bx
  - 15 Samples (+)
  - 41 Samples (-)
- Cytopenia at Bx
  - 8 Samples (+) 7 Samples (-)
- Cause of cytopenia
  - Identified: 5 Samples
  - Unidentified: 1 Sample
  - Identified: 18 Samples
  - Unidentified: 4 Sample
- Loss of osteoblasts
  - Partial:
    - 1 Sample
  - Complete:
    - 1 Sample
  - None:
    - 3 Samples

56 Samples
Figure 3

(A) BM Bx Samples day 100~

- cGVHD at Bx
  - (+) 19 Samples
  - (-) 14 Samples

- Cytopenia at Bx
  - (+) 16 Samples
  - (-) 3 Samples

- Cause of cytopenia
  - Identified 2 Samples
  - Unidentified 14 Samples

- Loss of osteoblasts
  - Partial Complete
    - 1 sample None
    - 4 Samples
  - Complete
    - 1 Sample None
    - 2 Samples None

(B) BM Bx Samples day 100~

- Cytopenia (+) at Bx
  - 25 Samples

- Cause of cytopenia
  - Identified 6 Samples
  - Unidentified 19 Samples

- Loss of osteoblasts
  - (+) 2 Samples
  - (-) 4 Samples
  - None 12 Samples

- cGVHD
  - None 1 Samples
  - Limited 2 Samples
  - Extensive 7 Samples
Supplemental Table 1. BM cellularities and cytopenias in all samples

<table>
<thead>
<tr>
<th></th>
<th>Cytopenias (+) in PB</th>
<th>Cytopenias (-) in PB</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td><strong>BM cellularity</strong></td>
<td></td>
<td></td>
<td>0.0024</td>
</tr>
<tr>
<td>Median</td>
<td>20%</td>
<td>37.50%</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-90%</td>
<td>20-70%</td>
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Supplemental Table 2. Characteristics of GVHD and chimerism

<table>
<thead>
<tr>
<th>Duration from onset of aGVHD to BM Bx</th>
<th>Number of all samples</th>
<th>Median (Range)</th>
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<tbody>
<tr>
<td></td>
<td>15</td>
<td>8 days (0-77)</td>
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</table>

<table>
<thead>
<tr>
<th>Duration from onset of cGVHD to BM Bx</th>
<th>Number of all samples</th>
<th>Median (Range)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>19</td>
<td>68 days (0-322)</td>
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</table>

<table>
<thead>
<tr>
<th>Duration from onset of cGVHD to BM Bx</th>
<th>Number of samples with loss of osteoblasts</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
<td>63 days (0-177)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Donor chimerism (%) of samples with loss of osteoblasts</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% (2-100)</td>
</tr>
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Supplemental Table 3. Characteristics of patients and BM biopsy samples

<table>
<thead>
<tr>
<th>Timing of BM biopsy before transplantation (range)</th>
<th>Day -22 (Day -174 ~ Day -8)</th>
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<tbody>
<tr>
<td>Total numbers of samples collected after transplantation (median from Day 0 (range))</td>
<td>89 samples (Day 63 (Day 18 ~ Day 527))</td>
</tr>
<tr>
<td>Average numbers of BM harvest in a case (range)</td>
<td>1.7 times (1 ~ 6 times)</td>
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