conditioning regimen in our patient because of cardiac dysfunction. We used an alkylating agent thiotepa, since, at a standard dose level (10-30 mg/m$^2$), the dose-limiting toxicity is myelosuppression, and other toxicities are infrequent (Wolff et al. 1990). We reduced the dose of cyclophosphamide (50 mg/kg/day on days -3 and -2) in combination with thiotepa (10 mg/kg on day -5) as described by Aversa et al. (1999). The post-transplant course was uneventful. The 1-year follow-up showed a good response with improvement of skin manifestations and immunological abnormalities. Recently, Giorgetti et al. (2004) also described successful autologous PBSCT using a conditioning regimen containing thiotepa in a patient with SSc and cardiac involvement. Therefore, thiotepa is suitable for a conditioning regimen of auto-PBSCT in patients with cardiac dysfunction including SSc in order to reduce cyclophosphamide-induced cardiotoxicity.

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Letter to the Editor

# Vaginal Outflow Tract Obstruction Associated with Chronic Graft-versus-Host Disease Following Allogeneic Peripheral Blood Stem Cell Transplantation

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Gynecological abnormalities following allogeneic hematopoietic stem cell transplantation include reduced vaginal elasticity and rugal folds, small vaginal, uterine, and cervical size, atrophic vulvovaginitis, introital stenosis, and loss of pubic hair, symptoms that are attributed to secondary ovarian failure [1]. We report the unusual complication of vaginal outflow tract obstruction occurring after allogeneic blood stem cell transplantation for lymphoblastic lymphoma.

A 23-year-old Japanese woman was diagnosed with advanced lymphoblastic lymphoma with mediastinal bulky mass, bone marrow infiltration, and bilateral kidney involvement. Induction chemotherapy consisting of adriamycin, vincristine, and prednisolone achieved complete remission, and consolidation therapy was given. Six months after diagnosis, she received a conditioning regimen of total body irradiation and cyclophosphamide, followed by an infusion of granulocyte colony-stimulating factor (G-CSF) mobilized allogeneic blood stem cells containing $3.94 \times 10^6$/kg $CD34^+$ cells from her HLA-matched sister. Cyclosporine and short-term methotrexate were given for the prophylaxis of acute graft-versus-host disease (GVHD). Engraftment was prompt, and acute GVHD was not observed. Cyclosporine was gradually tapered. On day 107, she developed extensive chronic GVHD with a macropapular rash over the trunk and extremities, oral mucositis, and elevated levels of transaminases. A skin biopsy confirmed the diagnosis of GVHD. Her skin manifestation and abnormal liver function test responded to alternative administrations of cyclosporine and prednisolone. Eleven months after transplantation, when the abnormal liver function test was resolved, hormone replacement therapy with estrogen and progesterone was started for secondary ovarian failure. One month after starting hormone replacement therapy, she complained of lower abdominal pain with no menstrual discharge. Gynecological examination and ultrasound sonography demonstrated vaginal adhesion and hematocolpos. Hormone replacement therapy was stopped and the amount of accumulated blood in the vagina decreased. Immunosuppressive therapy with cyclosporine and prednisolone was given continuously for the treatment of chronic GVHD. Two years after transplantation, she got married and decided to undergo surgery for adhesiolysis.

Surgical treatment involved progressive dissection between the 2 faces of the vagina, followed by the placement of a vaginal dilator. The vaginal device was kept in place for 4 days, then she was instructed to withdraw it in the morning and replace it every night. The duration of hospitalization was 12 days postoperatively. One month after operation, the resumption of protected sexual intercourse was encouraged to maintain vaginal patency. She ceased to use the vaginal dilator at night 9 months postoperatively, and hormone replacement therapy was restarted. Her underlying disease has remained continuously in remission and she has no active chronic GVHD including skin, liver, oral, and lachrymal manifestations, without any immunosuppressive drugs. Vaginal patency has been maintained, but she still has some dyspareunia.

To date, 61 female patients have received allogeneic hematopoietic stem cell transplantation in our center, and this was the first patient with this complication. Vaginal stenosis is considered a rare complication following allogeneic hematopoietic stem cell transplantation [2-4], and chronic GVHD appears to be a risk factor for this genital abnormality [4-6]. This patient was receiving hormone replacement therapy, and therefore vulvar hypoestrogen lesions can be excluded. Spinelli et al have recently reported that genital manifestations of chronic GVHD are not unusual, and 53 of their 213 female patients (24.8%) showed clinical evidence of genital lesions that were considered an expression of GVHD [6]. They have also reported severe genital GVHD including vaginal adhesion or complete vaginal closure in 7 patients. Thus, genital manifestations can be associated with chronic GVHD, but patients may not report

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their symptoms. Early detection of genital GVHD might reduce the requirement for surgical intervention because local corticosteroids can be applied to counteract the inflammatory process of mild GVHD lesions [7]. Regular questioning and gynecological examinations are recommended for early detection of this complication.

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# Expression of the myeloperoxidase gene in AC133 positive leukemia cells relates to the prognosis of acute myeloid leukemia

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**Abstract**

We previously reported that the percentage of myeloperoxidase (MPO) positive blasts had a prognostic impact on survival of patients with acute myeloid leukemia (AML). To extend this observation, we quantitatively measured the level of the MPO gene in AC133 positive leukemia cells that would contain a putative AML stem/progenitor compartment. AML cases were divided into the MPO gene high (MPOg-H) and MPO gene low (MPOg-L) groups. Only patients belonging to the MPOg-H group had a favorable chromosomal translocation, t(8;21), and having no morphological dysplasia that was associated with MPOg-L. The difference in the survival of MPOg-H and MPOg-L was statistically meaningful, demonstrating the possible prognostic impact of the expression of MPO gene in AC133 positive leukemia cells.


*Keywords:* Myeloperoxidase; Gene expression; AC133; Acute myeloid leukemia; Prognostic factor

## 1. Introduction

Myeloperoxidase (MPO) is an enzyme exclusively expressed in hematopoietic cells committed to myeloid lineage [1–4]. Based on its specific expression in normal myeloid cells, both the enzymatic activity and the presence of MPO protein in leukemia blasts have been used for the diagnosis of acute myeloid leukemia (AML) by the French–American–British (FAB) group [5] as prime markers for the myeloid lineage of leukemia blasts.

Apart from its role in diagnosis, MPO in leukemia blasts was also shown to have a prognostic value by several groups [6–8]. In our recent report [8], AML patients with high percentage of MPO positive blasts (>50% of blasts are MPO activity positive, blast MPOa-H group) defined by routine cytochemical staining had a significantly better outcome compared to the low MPO activity positive blast group (MPO activity positive blasts $\leq$ 50%, blast MPOa-L). Multivariate analysis picked up the percentage of MPO positive blast as an independent prognostic factor along with karyotypes, WBC count at diagnosis and age. Considering that most of AML cases with favorable karyotyes, such as t(8;21) or inv(16) belong to the blast MPOa-H group (83 out of 88 cases in our previous report), it is suggested that MPO is one of the proteins highly expressed in leukemia blasts of AML cases with favorable prognosis by conventional chemotherapy.

*Abbreviations:* AML, acute myeloid leukemia; FAB, French–American–British; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLD, multilineage myelodysplasia; MPO, myeloperoxidase; PBS, phosphate-buffered saline; WBC, White blood cell

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Recent studies on leukemia cell populations revealed that a hierarchy of differentiation exists in AML blasts consisting of leukemia stem/progenitor cells and maturing blast cells. AML stem cells that are transplantable into NOD-SCID mice constitute a small proportion of leukemia cell population and they bear surface markers usually found on normal hematopoietic stem cells, such as CD34 and AC133 (CD133, PROMININ1 [PROM1]) [9–14]. CD133 expression has been demonstrated not only on hematopoietic stem cells shown by the reconstitution of hemtopoiesis using transplantation model but also on other tissue cells, such as undifferentiated epithelium and fetal brain neural stem cells. It is suggested that CD133 could be expressed on the surface of various stem/progenitor cells [15]. These antigens were successfully used to select leukemia stem cells to analyze, for example, the gene expression profile in hematopoietic stem cells or leukemia stem cells [9,14–17]. We have also examined the expression of more than 12,000 genes in AC133 positive leukemia cells, and compared the gene expression profiles between AML cases with and without morphological dysplasia (AML with multilineage dysplasia, AML/MLD and AML/non-MLD), demonstrating the different gene expression profiles in these two groups [18]. In this analysis, we also found that, in AC133 positive AML cells, MPO gene was expressed more in cases without dysplasia than those accompanied with dysplasia (Tsutsumi et al., unpublished data). Since AML/MLD tends to have a worse prognosis than AML/non-MLD [19], the expression of the MPO gene in AC133 positive cells seemed in accordance with the relationship between clinical outcome of AML and the percentage of MPO positive blasts judged by cytochemichal examination of bone marrow smears. These results suggested that the MPO gene would be expressed in an immature fraction of leukemia cells that contains leukemia stem cells, and that the expression level of the MPO gene in AC133 positive cells might be also related to prognosis.

With these backgrounds, we quantitatively measured the expression of the MPO gene in AC133 positive leukemia cells and we found that the level of MPO gene expression divided AML cases into two groups: MPO gene high (MPOg-H) and MPO gene low (MPOg-L).

We confirmed that all AML/MLD cases belonged to MPOg-L. We could also demonstrate the prognosis of MPOg-H group was better than MPOg-L, and that karyotypes related to poor prognosis were found only in MPOg-L. These findings suggested that the level of MPO gene expression in AC133 leukemia cells related to the prognosis of AML.

## 2. Materials and methods

### 2.1. Cell separation and purification

After obtaining written informed consent, bone marrow samples were collected from 33 patients with de novo AML before treatment and from 10 healthy volunteers as control.

Table 1
Percentage of AC133 positive cells before and after purification

| Case number | Before purification (%) | After purification (%) |
|---|---|---|
| 7 | 14 | 97.4 |
| 8 | 74.5 | 99.6 |
| 9 | 12.1 | 98.8 |
| 11 | 63 | 84.3 |
| 14 | 36.2 | 99.3 |
| 16 | 65.4 | 98.8 |
| 17 | 72.4 | 94.1 |

The method to purify AC133 positive cells was described previously [18]. Shortly, target cells were selected from bone marrow mononuclear cells with anti-AC133 antibody-conjugated magnetic microbeads and MACS magnetic separation columns (AC133 Isolation Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany).

When the number of AC133 positive cells was more than $1 \times 10^6$ after selection (seven cases, Table 1), the percentage of AC133 positive cells was assessed before and after purification using a flowcytometer (FACScan, Becton Dickinson, Oxford, UK) and anti-AC133/2 antibody (Miltenyi Biotec). In other cases, the isolated cells were morphologically examined on cytospin slides (May–Grunwald–Giemsa staining) to check the contamination of promyelocytes.

### 2.2. Quantitative real-time PCR (RT-PCR)

The expression of the MPO gene was assessed using quantitative real-time PCR method. cDNA was synthesized from total cellular RNA isolated from purified AC133 positive cells (using RNeasy mini, QIAGEN GmbH, Hilden, Germany) with oligo dT primer (ProSTARTM First-strand cDNA Kit, STRATAGENE, CA, USA), that was used as a template for the PCR reaction. RT-PCR was performed using LightCycler, SYBR Green System (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. The PCR was conducted for 40 cycles (95 °C for 15 s, 60 °C for 10 s and 72 °C for 7 s as one cycle) and 45 cycles to amplify the MPO gene and the GAPDH gene, respectively. The sequences of the PCR primer sets were as follows: for MPO gene, 5′-AACTGATGGAGCAGTATGGCACGC-3′ and 5′-TCGCTGCTGCATGCTGAACACACC-3′ and for the GAPDH gene, 5′-GTCAGTGGTGGACCTGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. Data of RT-PCR were standardized with the following control samples: cDNA of the U937 cell line for GAPDH (quantitative range, $10^0$ to $10^{-6}$) and MPO cDNA for MPO (quantitative range, $10^0$ to $10^{-6}$). In cases with chromosomal translocation between 8 and 21, AML1-ETO fusion transcript was also quantitatively assessed before and after the purification of AC133 positive fraction (primer sets for the AML1-ETO fusion gene, 5′-CACCTACCACAGAGCCATCAAA-3′ and 5′-ATCCACAGGTGAGTCTGGCATT-3′) [20]. All PCR reactions were performed at least twice. The amplification of target genes were confirmed by examining the melting

curves of products and by the electrophoresis of PCR products on a 2% agarose gel followed by the visualization with ethidium bromide staining.

### 2.3. Cell staining

In some cases, to show the presence of MPO protein and its enzymatic activity, AC133 positive cells spread on the slides were stained with anti-MPO antibody (Nichirei Corporation, Tokyo, Japan) using a DAKO LSAB + Kit (DAKO Corporation, CA, USA) and with the diaminobdnzidine method [21], respectively. The expression of MPO protein or its enzymatic activity was shown as a percentage of MPO (protein or activity) positive cells.

### 2.4. Cytogenetic risk group

Based on the karyotype of leukemia cells, patients were classified into either the favorable, intermediate or adverse risk group, defined by the MRC group with minor modification [22,23].

### 2.5. Statistical analyses

Clinical and hematological data were obtained from the medical record of each case. The comparison of multilineage dysplasia of hematopoietic cells in the presence of leukemia blasts [23] and the presence of Auer body between groups were analyzed using Chi-square test. The cytogenetic risk group was compared using Mantel extension test. WBC count and the percentage of MPO positive blasts among the groups were analyzed using Wilcoxon's rank sum test.

## 3. Results

### 3.1. Purification of AC133 positive cells

The percentage of AC133 positive leukemia cells in bone marrow varied from case to case (0.3–76.6% of mononuclear cells in 20 cases tested), and it did not have any relationship to the FAB subtypes of AML (data not shown) as reported previously [24,25]. After purification, an analysis with flowcytometer demonstrated that AC133 was positive in 84.4–99.6% of collected cells (median 98.8%) among seven cases in which we could obtained more than $1 \times 10^6$ cells (Table 1). Though the percentages of AC133 positive cells somewhat varied after purification, there was no differentiated myeloid cells, such as promyelocytes, under morphological evaluation of the slides.

Since some of normal stem cells have been shown to have AC133 antigen on its surface, we next assessed whether leukemia cells but not residual normal stem cells were selected by the purification procedure. For this purpose, we utilized cases with a specific chromosomal translocation, t(8;21), and the expression of AML1-ETO fusion gene resulting from this translocation was quantitatively measured after the purification. As shown in Table 2, the expression of AML1-ETO fusion gene was detected in all four cases though its level was distributed from 0.7 to 22.1. It demonstrated that the purified samples contained target leukemia cells in these four cases.

Table 2
Amount of AML1-ETO transcript after selection with AC133 columun

| Case number | AML1-ETO/GAPDH ratio |
|---|---|
| 3 | 7.77 |
| 4 | 22.1 |
| 10 | 0.7 |
| 11 | 4.88 |
| Negative control[a] | <0.01 |
| Kasumi-1 cell line[b] | 167.02 |

[a] M1 case with normal karyptype.
[b] No selection procedure.

### 3.2. Quantitative measurement of the MPO gene by real-time RT-PCR method

The relative amount of MPO transcript was shown as a ratio of the MPO and GAPDH transcripts (MPO/GAPDH ratio), and the data are summarized in Table 3. Among control samples, the MPO/GAPDH ratio ranged from 3.2 to 11.8, showing similar values. On the other hand, the MPO/GAPDH ratios varied widely in AML cases (0.05–49.9). Referring to the distribution of the ratios among AML samples and that of normal control (Table 3), we divided the AML cases into two groups: MPO gene high group (MPO/GAPDH ratio > 15, MPOg-H, 10 cases) and MPO gene low group (MPO/GAPDH ratio $\leqq$ 15, MPOg-L, 23 cases) so that all normal controls belonged to the MPOg-L group (Fig. 1). In some cases, MPO protein and its enzymatic activity were also examined on the cytospin slides of AC133 positive cells (Table 4). The enzymatic activity of MPO in AC133 positive

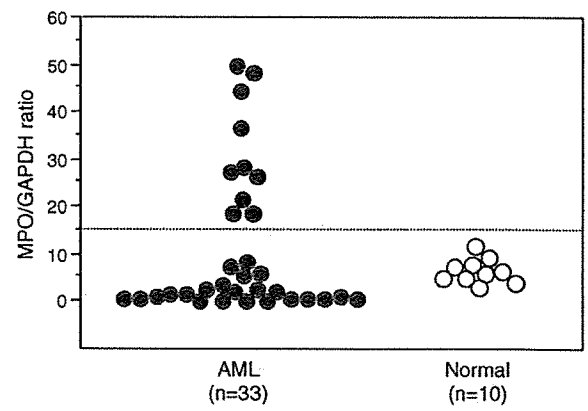


Fig. 1. Relative amount of MPO transcripts in AML cells (33 cases) and normal bone marrow cells (10 cases) selected with AC133-columns. The ratios of the MPO and GAPDH genes are shown as MPO/GAPDH ratio. AML cases were divided into two groups by the ratio (>15 and $\leqq$15). The dotted line shows the ratio, 15.

Table 3
MPO/GAPDH ratio and the percentage of MPO positive blasts

| Case number | MPO/GAPDH ratio | BM blast-MPO | FAB | MLD | Cytogenetic risk group | MPOg group |
|---|---|---|---|---|---|---|
| 1 | 7.37 | 50 | M2 | + | Adverse, 5q− | L |
| 2 | 5.37 | 33 | M6 | + | Adverse, −5 | L |
| 3 | 44.26 | 100 | M2 | − | Favorable, t(8;21) | H |
| 4 | 36.58 | 96 | M1 | − | Favorable, t(8;21) | H |
| 5 | 0.65 | 6 | M2 | + | Adverse, complex | L |
| 6 | 1.80 | 45 | M2 | + | Intermediate, normal | L |
| 7 | 2.83 | 35 | M2 | + | Intermediate, normal | L |
| 8 | 0.05 | 2 | M0 | − | Intermediate, others | L |
| 9 | 0.07 | 10 | M2 | − | Intermediate, normal | L |
| 10 | 21.46 | 100 | M2 | − | Favorable, t(8;21) | H |
| 11 | 18.77 | 100 | M2 | − | Favorable, t(8;21) | H |
| 12 | 2.35 | 13 | M4 | + | Adverse, complex | L |
| 13 | 0.12 | 4 | M4 | − | Intermediate, others | L |
| 14 | 0.60 | 3 | M6 | − | Adverse, complex | L |
| 15 | 49.91 | 80 | M1 | − | Intermediate, normal | H |
| 16 | 18.67 | 100 | M4 | − | Intermediate, others | H |
| 17 | 8.50 | 80 | M4-Eo | − | Favorable, inv(16) | L |
| 18 | 0.92 | 60 | M4 | + | Intermediate, others | L |
| 19 | 48.52 | 98 | M2 | − | Intermediate, others | H |
| 20 | 27.55 | 99 | M1 | − | Intermediate, normal | H |
| 21 | 28.34 | 94 | M2 | − | Intermediate, others | H |
| 22 | 2.09 | 26 | M2 | + | Intermediate, normal | L |
| 23 | 0.83 | 10 | M4 | − | Intermediate, normal | L |
| 24 | 2.43 | 59 | M2 | + | Adverse, −5 | L |
| 25 | 1.33 | 2 | M0 | − | Intermediate, normal | L |
| 26 | 3.78 | 80 | M2 | + | Intermediate, normal | L |
| 27 | 0.09 | 5 | M4 | + | Intermediate, normal | L |
| 28 | 26.48 | 100 | M1 | − | Intermediate, normal | H |
| 29 | 0.66 | 84 | M2 | − | Intermediate, normal | L |
| 30 | 1.11 | 45 | M2 | + | Adverse, complex | L |
| 31 | 0.53 | 11 | M2 | + | Intermediate, normal | L |
| 32 | 1.58 | 54 | M4 | + | ND | L |
| 33 | 5.95 | 82 | M2 | + | Intermediate, normal | L |
| Normal 1 | 11.8 | ND | | | | |
| Normal 2 | 9.5 | ND | | | | |
| Normal 3 | 8.0 | ND | | | | |
| Normal 4 | 7.5 | ND | | | | |
| Normal 5 | 6.5 | ND | | | | |
| Normal 6 | 6.2 | ND | | | | |
| Normal 7 | 5.2 | ND | | | | |
| Normal 8 | 5.0 | ND | | | | |
| Normal 9 | 4.1 | ND | | | | |
| Normal 10 | 3.2 | ND | | | | |

ND, not done; MLD, multilineage dysplasia.

cells was detected mostly in cases belonging to MPOg-H group, and they had high percentages of MPO protein positive cells except for case 33 (Table 4).

We next compared the level of expression of the MPO gene in AC133 positive cells and the percentage of MPO positive leukemia blasts judged on bone marrow slides. The relationship of these two factors is shown in Fig. 2. Cases were classified into the high percentage of MPO (activity) positive blasts (blast MPOa-H group, MPO activity positive blasts > 50%) or the low group (blast MPOa-L group, MPO activity positive blasts $\leqq$ 50%). All cases in the blast MPOa-L group were categorized into MPOg-L (Group III), however, the blast MPOa-H cases comprised of MPOg-H (Group I, 10 cases) and MPOg-L (Group II, 8 cases). It meant that cases in Group II showed high percentage of MPO positive blast on the bone marrow smear but the expression of the MPO gene was low in the AC133 positive fraction. The percentage of MPO positive blasts did not have a statistically significant prognostic impact on overall survival in this series (Fig. 3) but all long-term survivors belonged to the MPOg-H group.

### 3.3. Clinical characteristics of cases in MPOg-H and MPOg-L groups

Clinical characteristics of AML cases in the MPOg-H and MPOg-L groups are shown in Table 5. There was no statistical difference in age or performance status (PS) among these two groups, however, multilineage morphological dysplasia

Table 4
Percentages of MPO protein or MPO activity positive cells among AC133 purified samples

| Case number | MPO/GAPDH ratio | MPOg group | AC133-MPO protein positive cell (%) | AC133-MPO activity positive cell (%) |
|---|---|---|---|---|
| 13 | 0.12 | L | 0 | 0 |
| 14 | 0.60 | L | <1 | 0 |
| 17 | 8.50 | L | 13 | 0 |
| 18 | 0.92 | L | 10 | 0 |
| 19 | 48.52 | H | 100 | 12 |
| 20 | 27.55 | H | 100 | 60 |
| 21 | 28.34 | H | 100 | 29 |
| 23 | 0.83 | L | 0 | 0 |
| 24 | 2.43 | L | 3 | 0 |
| 25 | 1.33 | L | 0 | 0 |
| 26 | 3.78 | L | 11 | 0 |
| 28 | 26.48 | H | 100 | 64 |
| 31 | 0.53 | L | 0 | 0 |
| 32 | 1.58 | L | 0 | 0 |
| 33 | 5.95 | L | 47 | 5 |

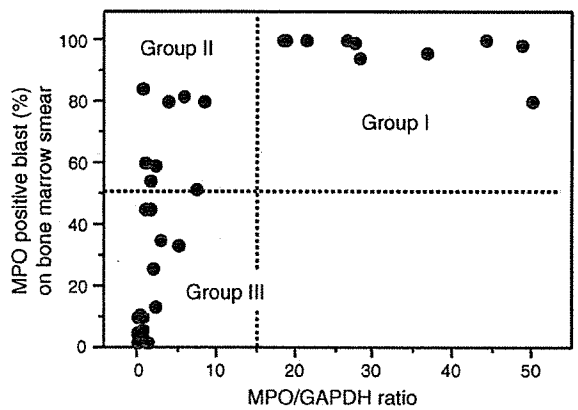


Fig. 2. Relationship between the percentage of MPO positive blasts on bone marrow smear and the amount of the MPO gene in AC133 positive cells. AML cases were categorized into three groups (Groups I–III) by the two factors above. The vertical dotted line shows the MPO/GAPDH ratio, 15, and the horizontal one is for the percentage of MPO positive blasts, 50%. There were 10 cases in Group I, 8 cases in Group II and 15 cases in Group III.

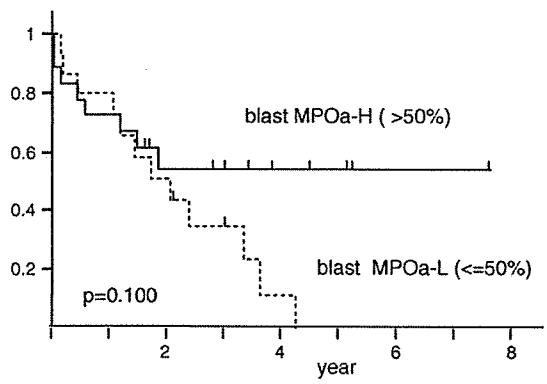


Fig. 3. Overall survival by the percentage of MPO positive blasts on bone marrow smear. There was no statistical significance between the high MPO group (blast MPOa-H) and the low MPO group (blast MPOa-L). The $p$-value was 0.100.

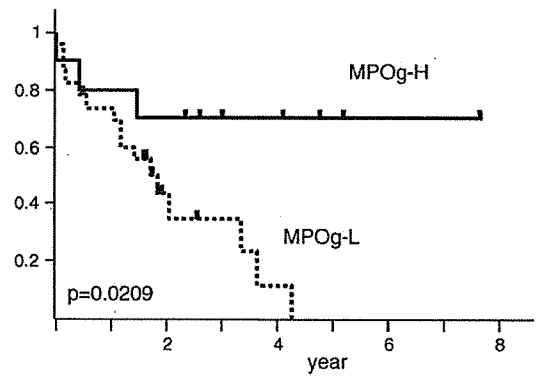


Fig. 4. Overall survival by the amount of MPO gene expression in AC133 positive cells. AML cases with high expression of the MPO gene in AC133 positive cells (MPOg-H) showed better overall survival than those with low expression (MPOg-L) with statistical significance ($p = 0.0209$).

(MLD) in the presence of leukemia blasts was found only in the cases belonging to the MPOg-L group. Distribution of cases in the chromosomal risk groups was also significantly different between MPOg-H and MPOg-L; four out of five cases with favorable karyotypes were in the MPOg-H, and all seven cases with adverse chromosomal risk belonged to the MPOg-L group. Interestingly, although the WBC count at diagnosis was significantly high in the MPOg-H group, overall survival was better in the MPOg-H group than the MPOg-L with statistical significance (Fig. 4).

## 4. Discussion

Recent reports have demonstrated that AC133 positive bone marrow/cord blood cells are capable of reconstituting long-term hematopoiesis both in mouse and man [12,13,26,27], and that human AML blasts bearing AC133 are able to proliferate and form a leukemic cell population in NOD-SCID mice [11]. From these reports, it has been suggested that normal and malignant hematopoietic stem cells

Table 5
Clinical characteristics of patients in the high and low MPO/GAPDH group

| | Total ($n=33$) | High ($n=10$) | Low ($n=23$) | $p$-Value |
|---|---|---|---|---|
| Age, Median (range) | | 34 (27–79) | 52 (22–85) | 0.0958 |
| PS (0/1/2/3) | 5/23/2/3 | 2/7/1/0 | 3/16/1/3 | 0.3445 |
| FAB type | | | | 0.0418 |
| M0 | 2 | 0 | 2 | |
| M1 | 4 | 4 | 0 | |
| M2 | 17 | 5 | 12 | |
| M4 | 8 | 1 | 7 | |
| M6 | 2 | 0 | 2 | |
| WBC, median ($\times 10^9\,l^{-1}$) | 16.5 | 58.0 | 9.9 | 0.0092 |
| Auer body (present/absent) | 14/19 | 9/1 | 5/18 | 0.0003 |
| MPO positivity of blast, median (%) | 52 | 99 | 34 | <0.0001 |
| MLD (present/absent) | 15/18 | 0/10 | 15/8 | 0.0005 |
| Cytogenetic risk group | | | | 0.0044 |
| Favorable | 5 | 4 | 1 | |
| Intermediate | 20 | 6 | 14 | |
| Adverse | 7 | 0 | 7 | |
| Not done | 1 | 0 | 1 | |

MLD, multilineage dysplasia.

were positive for AC133 antigen on the surface. In this study, we measured the amount of MPO transcripts in AC133 positive leukemia cells. As shown in cases with t(8;21), the expression of AML1-ETO fusion transcripts was detectable after positive selection with an AC133-column, demonstrating these four purified samples, at least, contained leukemia cells. Morphological examination showed that the selected cells did not contain any promyelocytes or more mature cells.

The expression of the MPO gene in AML cells was previously reported by several groups [28–30]. Zaki et al. used Northern blot analysis to examine the expression of MPO mRNA in 32 AML samples, and they found that M3 cases had the highest level of expression followed by M2, M4, M1 and M5 cases [28]. Since their results reflected the myeloid differentiation of leukemia cells defined by the FAB subtypes, it seemed that the AML samples they tested (MNC samples, >80% blasts) contained a differentiated fraction of leukemia cells. In our present study, AML with maturation, such as M2 and M4 cases were found in both MPOg-H and MPOg-L groups, showing a clear contrast to the report from Zaki et al. We assume that the amount of MPO mRNA in AC133 positive leukemia cells did not clearly relate to the FAB subgroup of AML defined by the morphological differentiation of leukemia cells.

It has been shown that genes expressed in immature hamatopoietic cells including stem cells do not always represent the lineage commitment [31,32]. Using genetically engineered mice, Ye et al. demonstrated that one of the myeloid specific genes, lysozyme, was expressed in bone marrow cells that have potential to reconstitute both myeloid and lymphoid cells [33]. We do not have any clear answer whether the expression of the MPO gene in AC133 positive leukemia cells was independent of myeloid commitment or it was a part of myeloid differentiation of these cells. The fact that some cells in cases belonging to MPOg-H also expressed MPO protein and its enzymatic activity suggested, at least in these cases, that MPO expression represented the early process of myeloid commitment and/or maturation before apparent morphological differentiation.

Comparing to the MPOg-L, belonging to the MPOg-H group was significantly related to the better survival. Some clinical features repeatedly observed in favorable AML cases were found among cases in the MPOg-H group: all cases with t(8;21) and no cases with adverse karyotypes or MLD were in this group [34]. These data supported the difference in survival between these two groups. We previously demonstrated that the percentage of MPO positive blasts was an independent prognostic factor for AML [8]. However, there was no significant difference in overall survival by the percentage of MPO positive blasts in this study ($p=0.100$), but by the level of the MPO gene in AC133 positive fraction. It might be because of the small number of cases, or it was because the level of the MPO gene in AC133 positive fraction might have a stronger impact on the survival of patients with AML. Survival curve of Group II (high percentage of MPO positive blasts but low MPO/GAPDH ratio) was similar to that of Group III in this series (data not shown). This point needs to be confirmed with a larger number of cases.

In summary, we confirmed that MLD phenotype was significantly related to the low expression of the MPO gene in AC133 positive cells. We also demonstrated the possible prognostic value of the MPOg-H group in overall survival associated with positive relation to the karyotype, t(8;21) and the negative relation to the adverse karyotypes. It is necessary to investigate whether several factors seen in favorable AML cases, such as karyotype and MPO expression have biological relationship at the molecular level.

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IMMUNOBIOLOGY

# Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia

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**To identify novel autoantibodies in acquired aplastic anemia (AA), we screened the sera of patients with AA possessing small populations of paroxysmal nocturnal hemoglobinuria (PNH)–type cells for the presence of antibodies (Abs) which recognize proteins derived from a leukemia cell line, UT-7. Immunoblotting using proteins derived from lysates or culture supernatants of UT-7 cells revealed the presence of IgG Abs specific to an 80-kDa protein. Peptide mass fingerprinting identified this 80-kDa protein as moesin. Enzyme-linked immunosorbent assay (ELISA) using recombinant moesin showed high titers of antimoesin Abs in 25 (37%) of 67 patients with AA. Moesin was secreted from several myeloid leukemia cell lines other than UT-7, such as OUN-1 and K562, as an exosomal protein. The presence of antimoesin Abs was significantly correlated with the presence of PNH-type cells and antidiazepam-binding inhibitor-related protein-1 (DRS-1) Abs. Patients with AA that did not show any of these 3 markers tended to respond poorly to immunosuppressive therapy. These findings suggest that a B-cell response to moesin, possibly derived from hematopoietic cells, frequently occurs in patients with AA and that detection of antimoesin Abs in combination with other markers may be useful in diagnosing immune pathophysiology in patients with AA. (Blood. 2007;109:000-000)**



## Introduction

Acquired aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Immune-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome,[1-3] but this mechanism does not underlie all patients with AA. Approximately 30% of patients with AA do not respond to immunosuppressive therapy (IST) with anti–thymocyte globulin (ATG) plus cyclosporine (CsA),[4,5] and some of the patients treated with IST undergo progression to myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML). For patients with nonimmune mediated AA, IST might even be harmful because of an increased risk of opportunistic infections. Despite the understanding that not all patients with AA have an immune pathophysiology, patients with AA have been unconditionally placed on IST because there is no good marker to differentiate nonimmune-mediated AA from immune-mediated AA.

We recently reported that the presence of a small population of $CD55^-CD59^-$ blood cells serves as a marker for immune pathophysiology of AA.[6-8] However, the highly sensitive flow cytometric assay which determines whether there is an increase in the proportion of such paroxysmal nocturnal hemoglobinuria (PNH)–type cells requires fresh blood. The assay to detect PNH-type granulocytes is difficult to use on patients with AA whose neutrophil counts are less than $0.1 \times 10^9$/L. Moreover, even in the absence of an increased proportion of PNH-type cells, greater than 40% of patients with AA respond to IST.[8] Accordingly, laboratory markers other than small populations of PNH-type cells are necessary to diagnose immune pathophysiology prior to IST in patients with AA.

In autoimmune diseases in which T cells play a primary role in the pathogenesis, antibodies (Abs) specific to antigens derived from T-cell target proteins, such as myelin basic protein in multiple sclerosis (MS) and glutamate decarboxylase in insulin-dependent diabetes mellitus (IDDM), are often detected, and they have served as a marker of immune pathophysiology of the diseases.[9-12] If Abs specific to the antigens abundantly expressed by hematopoietic progenitor cells were detectable in the serum of patients with AA, they might reflect the immune pathophysiology of their patients. We previously identified Abs specific to diazepam-biding inhibitor-related protein-1 (DRS-1) in the serum of 38% of patients with AA showing small populations of PNH-type cells (patients with $PNH^+$ AA).[13] The serum of patients with $PNH^+$ AA may also contain other autoantibodies (auto-Abs) specific to self-antigens because of a breakdown of tolerance that is thought to occur in organ-specific autoimmune diseases such as MS,[14] IDDM,[15] and Basedow disease.[16] The detection of such Abs may complement the diagnosis of immune pathophysiology in patients with AA.

To identify novel auto-Abs in AA, we screened sera from patients with $PNH^+$ AA for the presence of Abs recognizing antigens derived from a megakaryocytic leukemia cell line, UT-7, a cDNA library of which was used for the identification of anti–DRS-1 Abs.[13] A new method taking advantage of culture supernatant of the cells for Western blotting followed by mass spectrometry identified an Ab specific to a membrane-cytoskeleton linking protein, moesin.

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## Patients, materials, and methods

### Study subjects

AQ: 6 Sera or plasma were obtained from 67 patients with aplastic anemia (AA) (23 with severe AA and 44 with moderate AA), 21 patients with refractory AQ: 7 anemia (RA) of myelodysplastic syndrome (MDS), 49 patients with rheumatoid arthritis, and 48 healthy individuals. The severity of AA was classified according to the criteria proposed by Camitta et al.[17] Samples were cryopreserved at −80°C until use. All patients and healthy volunteers were provided informed consent in accordance with the Declaration of Helsinki, before collecting samples. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science. AA was diagnosed in patients at Kanazawa University Hospital and other hospitals taking part in the bone marrow failure study led by the Ministry of Health, Labor, and Welfare of Japan.

### Detection of PNH-type cells

Proportions of $CD55^-CD59^-$ cells in $CD11b^+$ granulocytes and in AQ: 8 glycophorin $A^+$ red blood cells (RBCs) were determined using 2-color flow cytometry as described previously.[8]

### Cell lines

The following leukemia cell lines and synovial cells were kindly provided by each respective researcher. A megakaryoblastic leukemia cell line UT-7 by Dr N. Komatsu of Jichi Medical School; myeloid leukemia cell lines KH88, OUN-1, SAS413, NB4, and KG-1 by Dr M. Yasukawa of Ehime University; a myelodysplastic syndrome cell line TF-1 by Dr S. Ogawa of the University of Tokyo; synovial cells of a patient with rheumatoid arthritis by Dr M. Kawano of Kanazawa University. K562 was purchased from the Health Science Research Resources Bank (Osaka, Japan).

### Isolation of $CD34^+$ cells, $CD3^+$ T cells, and $CD19^+$ B cells

Peripheral blood buffy coat cells were collected from a healthy volunteer donor after G-CSF administration. $CD34^+$ cells were isolated from the buffy coat cells using a CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD3^+$ T cells and $CD19^+$ B cells were isolated from the same cells using CD3 and CD19 microbeads (Miltenyi Biotec), respectively. The purity of isolated $CD34^+$, $CD3^+$, and $CD19^+$ cells was greater than 95% as demonstrated by flow cytometry.

### Immunofluorescence analysis of UT-7 cells

UT-7 cells were fixed with 4% formaldehyde for 5 minutes and treated with 0.1% Tween 20 for 10 minutes for permeabilization. The cells were incubated in PBS containing 0.5% sera from a patient with AA for 30 minutes. After washing, the cells were incubated in PBS containing 0.1% FITC-labeled goat anti–human IgG and viewed with an immunofluorescent microscope (Zeiss, Axioplan2 imaging).

### Purification of bacterially expressed moesin proteins

Full-length human moesin cDNA and the cDNA fragments of human moesin were synthesized by the reverse transcription–polymerase chain AQ: 9 reaction (RT-PCR) amplification method. Briefly, cDNA was reverse transcribed from the mRNA of UT-7 cells by using SuperScript First-Strand AQ: 10 Synthesis (Invitrogen, Carlsbad, CA). Thirty cycles of PCR were performed using each set of primers; for the full-length moesin termed M0 (S0, 5′-CGGAATTCGCCTTTGCCGCCACCATGCCC-3′; A0, 5′-CGGTCGACTCCCTAGAGGCTGGGTGCCCA-3′), for the carboxyl (C)–terminal portion of moesin (moesin 399-500) termed M1 (S1, 5′-CGCGGATCCGCCAAGGAGGCCTTGCTGCAG-3′; A1, 5′-CGGAATTCGGCCATAGCATCAGCCCGTAG-3′), and for the C-terminal portion of moesin (moesin 494-577) termed M2 (S2, 5′-CGCGGATCCCTACGGGCTGATGCTATGGCC-3′; A2, 5′-CGGAATTCTCCCTAGAGGCTGGGTGCCCA3-′). Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. PCR products purified from an agarose gel were inserted into the pGEX-6P-1 vector (GE Healthcare, Fairfield, CT) between the *Eco*RI and *Sal*I sites for expression AQ: 11 of a GST-tag fusion protein M0, and between the *Eco*RI and *Bam*HI sites for the GST-tag fusion proteins M1 and M2 using BL21 competent cells (Novagen, Madison, WI). Synthesized proteins were purified using gluta- AQ: 12 thione Sepharose 4B (Amersham Biosciences, Piscataway, NJ). Native AQ: 13 moesin protein and C-terminal moesin protein fragments were released from GST-tag moesin proteins using PreScission Protease (Amersham Biosciences) according to the manufacturer's instructions. The proper size of the recombinant proteins was confirmed by Western blotting with mouse antimoesin mAb (Clone 38; BD Biosciences, San Jose, CA). To detect a AQ: 14 specific Ab in serum, blotted membranes were incubated in 3% BSA-PBS containing serum diluted 1:200.

### Western blotting

UT-7 cells were suspended in Laemmli sample buffer and sonicated for the preparation of cell lysates. Approximately 10 μg UT-7 lysate, 5 μg Grp78, and 5 μg recombinant native moesin protein per lane were electrophoresed in a 12% polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated in 3% BSA-PBS containing serum diluted 1:200 from patients with AA or healthy persons.

### Peptide mass fingerprinting

UT-7 cells ($\times 10^7$) were suspended in 1 mL RPMI 1640 medium containing 10% fetal calf serum (FCS) and incubated for 1 hour at 37°C in the $CO_2$ incubator. Culture supernatants were collected by centrifugation at 500*g* for 5 minutes. Mass spectrometric identification of an 80-kDa protein derived from the UT-7 cell supernatants was performed as previously described.[18] Briefly, proteins fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were visualized by Coomassie Brilliant AQ: 15 Blue (CBB) staining, and the 80-kDa band was excised from gels, followed by in-gel digestions with trypsin (Promega, Madison, WI) in a buffer AQ: 16 containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the triptic peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using an ultraflex TOF/TOF (Bruker Daltonics, Billerica, MA). Proteins were identified by comparison between AQ: 17 the molecular weights determined by MALDI-TOF/MS and the theoretical peptide masses of proteins registered in NCBInr.

### Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well Nunc-Immuno plate (Nalge-Nunc, Roskilde, Denmark) was filled with 50 μL coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) containing 5 μg/mL purified recombinant moesin protein and was incubated overnight at 4°C. The plates were washed and filled with PBS containing 10% FCS for 2 hours at 37°C to block nonspecific binding of proteins to moesin. Sera from the patients were added to the wells at a final dilution of 1:200, and the plates were incubated at 37°C for 1 hour. After washing, peroxidase-conjugated goat anti–human IgG antibodies (1:80 000; Jackson ImmunoResearch, Baltimore, PA) were added to the wells, and the plates were incubated at 37°C for 1 hour. Finally, the plates were washed and incubated with 3,3,5,5-tetramethylbenzidine substrate (Pierce, Rockford, IL) at room temperature for 30 minutes, and the optical density (OD) absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments, Grödig, Austria). A positive reaction was defined as an absorbance value exceeding the mean +2 SDs of the OD absorbance values from the sera of the 20 or 48 controls.

### Enrichment of exosomes from culture supernatants of leukemia cell lines[19] and Western blotting AQ: 18

Leukemia cell lines (K562, OUN-1, TF-1, and UT-7) cells, $CD34^+$ cells, $CD3^+$ T cells, $CD19^+$ B cells, and synovial cells were cultured at $2 \times 10^9$/L for 48 hours, and the supernatants were collected. The culture supernatants of leukemia cell lines and synovial cells were subjected to 3 successive

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centrifugations to remove cells and cell debris at 300*g* for 10 minutes, 2000*g* for 20 minutes, and finally at 10 000*g* for 30 minutes, all at 4°C. Exosomes were then pelleted at 64 000*g* for 100 minutes. Pellets were resuspended and washed in PBS and centrifuged at 100 000*g* for 1 hour using a SW28 rotor (Beckman Coulter Instruments, Fullerton, CA). The exosomes were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer of pH 7.4 for 1 hour and then were postfixed with 0.5% $OsO_4$ for 20 minutes at 4°C. Next, they were stained with 0.5% uranyl acetate for 20 minutes, dehydrated with a graded ethanol series, and were embedded in an epoxy resin (Selva Feinbiochemica GmbH, Heidelberg, Germany). Ultrathin sections were prepared and examined using an electron microscope (JEM-1210; JEOL, Tokyo, Japan) after brief staining with uranyl acetate and lead citrate. Exosomes were resuspended in PBS, divided into aliquots, and stored at −80°C. Approximately 1 μg exosomal protein per lane was electrophoresed in a 12% polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated in PBS containing a mouse antimoesin mAb (Clone 38/87; NeoMarkers, Fremont, CA) at 0.2 μg/mL or a rabbit antimoesin polyclonal Ab (TK89) which was kindly provided by Dr S. Tsukita of Kyoto University.

### Immunosuppressive therapy and response criteria

Antithymocyte globulin [ATG; Lymphoglobuline; Aventis Behring, King of Prussia, PA; 15 mg/(kg/day), 5 days] plus cyclosporine [CsA; Novartis, Basel, Switzerland; 3-6 mg/kg/day)], or CsA alone was administered following the standard protocol. The dose of CsA was adjusted to maintain trough levels between 150 and 250 ng/mL, and the appropriate dose was administered for at least 6 months. G-CSF (filgrastim; 300 μg/m² or lenograstim 5 μg/kg) was administered to some patients. Response to IST was evaluated according to the response criteria described by Camitta.[20]

### Statistics

Differences in the prevalence of antimoesin Ab titers in serum among different patient groups were examined using Fisher exact test.

## Results

### Detection of Abs specific to proteins derived from UT-7 cells in the sera of patients with AA

We first screened sera of patients with AA showing increased PNH-type cells for the presence of Abs reactive to UT-7 cell proteins using an immunofluorescence analysis. Among the sera from 9 patients with AA, 6 patients' sera stained the cytoplasm of UT-7 cells as shown in Figure 1A, indicating the presence of IgG antibodies specific to UT-7 proteins. To identify these proteins, UT-7 lysate was subjected to Western blotting using sera from patients with AA. Sera from several patients with PNH⁺ AA produced a clear band with a size of 80 kDa (Figure 1B). The Ab specific to the 80-kDa protein was detected in the sera of 9 (43%) of 21 patients with PNH⁺ AA ,whereas it was undetectable in any of the 7 patients not showing increased PNH-type cells (PNH⁻ patients) and 11 healthy persons.

### Identification of the 80-kDa protein

To identify the 80-kDa protein, we cut out the clear band from a CBB-stained gel and tried to determine the amino acid sequence of the protein using peptide mass fingerprinting. However, this attempt failed to identify a single protein because of the presence of various other proteins in the total amount eluted from the 80-kDa band. We then examined culture supernatants of UT-7 cells for the presence of the 80-kDa protein because some intracellular proteins are reportedly secreted from cell lines into culture medium.[19,21,22] Figure 2 shows the results of Western blotting. Incubation of the membrane blotted with the culture supernatant proteins in PBS containing sera from patients with AA revealed a similar but more distinct 80-kDa band. When the corresponding band was cut out from the original CBB-stained gel (Figure 2, lane 5) and subjected to peptide mass fingerprinting, this protein was identified as either moesin or Grp 78. Serum of patients with AA failed to reveal an 80-kDa band when a Grp 78 protein blotted membrane was used. The approximately 70-kDa bands in lanes 3 and 6 of Figure 2 were thought to be nonspecific bands because of a low purity of the recombinant Grp 78 protein.

### Prevalence of antimoesin Abs in patients with AA or MDS

To confirm the presence of Abs specific to moesin in the sera of patients with AA, a recombinant human native moesin protein was prepared. Figure 3A shows the results of Western blotting using the native moesin. Clear bands corresponding to the moesin were produced by sera of 3 patients with PNH⁺ AA but not by sera from 2 healthy persons. When we used the patient serum at different dilutions of more than 1:200, including 1:400, 1:800, 1:1600, 1:6400, and 1:25 600, the serum diluted up to 1:6400 could detect moesin by Western blotting. To measure the titers of moesin Abs in the serum, we established an ELISA using recombinant moesin protein. Higher antimoesin Ab titers than the cutoff value were detected in 25 (37%) of 67 patients. The prevalence was 44% in 41 patients whose duration of illness was shorter than 1 year. Figure 3B shows titers of antimoesin Ab in the sera of 67 patients with AA consisting of 43 patients with PNH⁺ and 24 patients with PNH⁻, 21 patients with MDS-RA

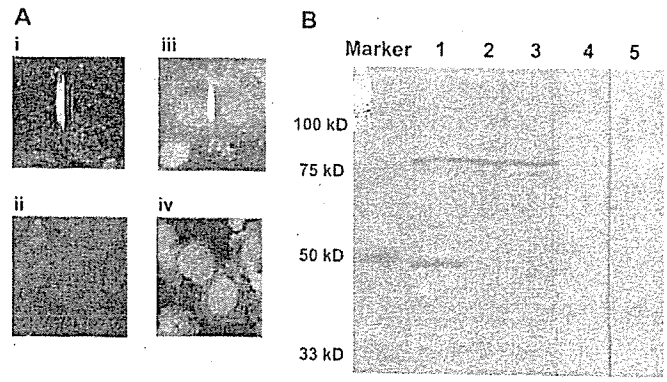


**Figure 1. Detection of antibodies (Abs) specific to proteins derived from UT-7 cells in the sera of patients with AA.** (A) Immunofluorescence analysis of UT-7 cells using 1:200 diluted sera and FITC-labeled anti–human IgG. (i-ii) Healthy persons; (iii-iv) patients with AA. (B) Western blotting for UT-7 lysates with sera from 3 patients with PNH⁺ AA (lanes 1-3) and 2 healthy persons (lanes 4-5).

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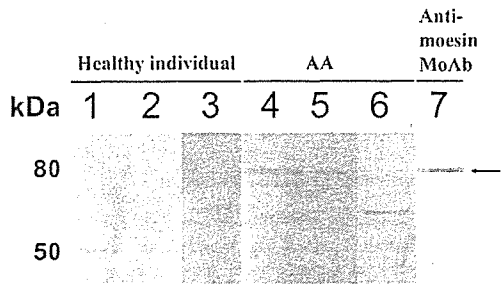


**Figure 2. Identification of the 80-kDa protein recognized by IgG Abs of patients with AA.** UT-7 lysates (lanes 1, 4), culture supernatants of UT-7 cells (lanes 2, 5, 7), and recombinant Grp 78 protein (lanes 3, 6) were subjected to Western blotting using sera from a healthy person (lanes 1-3), a patients with PNH$^+$ AA (lanes 4-6), and antimoesin monoclonal Ab (lane 7).

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consisting of 14 patients with PNH$^+$ and 7 patients with PNH$^-$, and 48 healthy persons. Twenty (47%) of the patients with PNH$^+$ AA showed Ab titers greater than the cutoff value, whereas 5 (21%) of the patients with PNH$^-$ AA showed increased titers. There was a significant difference in the prevalence of higher antimoesin Ab titers between patients with PNH$^+$ AA and patients with PNH$^-$ AA ($P$ = .03). Nine (64%) of the patients with PNH$^+$ MDS-RA showed Ab titers greater than the cutoff value, whereas 3 (43%) of the patients with PNH$^-$ MDS-RA showed increased titers. No significant difference was observed in the prevalence of higher antimoesin Ab titers between patients with PNH$^+$ MDS-RA and patients with PNH$^-$ MDS-RA ($P$ = .32). Among patients with recently diagnosed AA examined before therapy, the prevalence of patients showing higher antimoesin Ab titers than the cutoff value in 31 patients with PNH$^+$ (52%) was also higher than that in 10 patients with PNH$^-$ (20%), although the difference in the prevalence between the 2 groups was not statistically significant ($P$ = .08; Figure 3C). None of the sera from 6 patients undergoing chemotherapy (3 with acute myelogenous leukemia and 3 with non-Hodgkin lymphoma) were positive for antimoesin Abs.

### Epitope analysis of antimoesin Abs in patients with AA and those with rheumatoid arthritis

A previous study demonstrated that antimoesin Abs were detectable in 14% of patients with rheumatoid arthritis.[23] Our ELISA revealed high titers of antimoesin Abs in 34% of patients with rheumatoid arthritis who did not show any cytopenias (data not shown). To determine whether a difference exists in the specificity of antimoesin Abs between patients with AA and rheumatoid arthritis, 2 different C-terminal fragments of moesin (M1 and M2) were prepared and used for the ELISA. When we screened the sera from 3 patients showing high titers of antimoesin Abs against the full-length moesin for the presence of Abs specific to N-terminal proteins (amino acid residues 1-398) using Western blotting, none of the sera from these patients revealed a band of the N-terminal protein (data not shown). We therefore focused on the C-terminal protein to detect specific Abs with ELISA. As shown in Figure 3D-E, 12 (19%) patients with AA and 10 patients (20%) with rheumatoid arthritis showed anti-M1 Ab titers, whereas 11 (18%) patients with AA and 5 patients (10%) with rheumatoid arthritis showed anti-M2 Ab titers greater than the cutoff value. Therefore, the 2 groups of patients showed a similar pattern of Ab titers to each fragment.

### Secretion of moesin from leukemia cell lines, CD34$^+$ cells, CD3$^+$ T cells, CD19$^+$ B cells, and synovial cells

The secretion of moesin may be common to immature myeloid leukemia cells. We therefore examined culture supernatants of myeloid leukemia cell lines other than UT-7 cells as well as of CD34$^+$ cells, CD3$^+$ T cells, and CD19$^+$ B cells from a healthy volunteer donor. Moesin was detectable in culture supernatant of 4 (K562, OUN-1, NB4 and UT-7) of 7 myeloid leukemia cell lines, CD34$^+$ cells, and CD19$^+$ B cells (Figure 4A). Because B lymphocytes reportedly secrete moesin as a form of exosome,[24] we examined the exosomal fraction of culture supernatant derived from leukemia cell lines and synovial cells of a patient with rheumatoid arthritis for the presence of moesin. As shown in Figure 4B, moesin was detectable in the exosome fraction of culture supernatants from all 4 leukemia cell lines and synovial cells.

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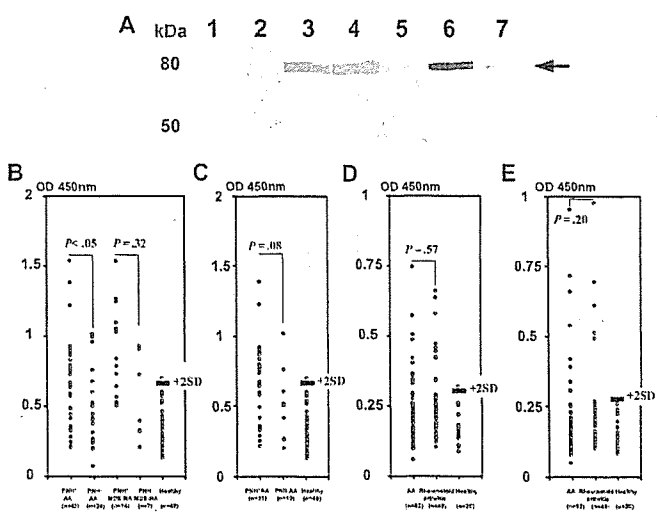


**Figure 3. INSERT TITLE HERE.** (A) Detection of specific Abs to recombinant moesin in the sera of patients with AA. Purified recombinant native moesin protein was used to detect Abs specific to moesin in sera from 2 healthy persons (lanes 1-2) and 3 patients with PNH$^+$ AA (lanes 3-5). Antimoesin monoclonal Ab was used as a positive control (lane 6). The serum of a patient with PNH$^+$ AA diluted to 1:6400 was also used (lane 7). (B-C) Titration of moesin Abs in sera of patients with AA and MDS-RA using ELISA. Antimoesin Ab titers were determined for the sera of 43 patients with PNH$^+$ AA, 24 patients with PNH$^-$ AA, 14 patients with PNH$^+$ MDS-RA, 7 patients with PNH$^-$ MDS-RA, and 48 healthy persons. (B) All patients. (C) Patients with recently diagnosed AA examined before therapy. (D-E) Titration of Abs specific to C-terminal moesin fragment in the sera of patients with AA and rheumatoid arthritis. Titers of anti–C-terminal moesin fragment Abs were determined in the sera of 62 patients with AA, 49 patients with rheumatoid arthritis, and 20 healthy persons. (D) Antimoesin C-terminal fragment (399-500) M1 Abs. (E) Antimoesin C-terminal fragment (494-577) M2 Abs. The solid line denotes a cutoff value defined as the mean + 2 SDs of the absorbance in healthy persons. $P$ values indicate the differences in the prevalence of patients showing higher antimoesin or moesin-fragment Ab titers between 2 different groups.

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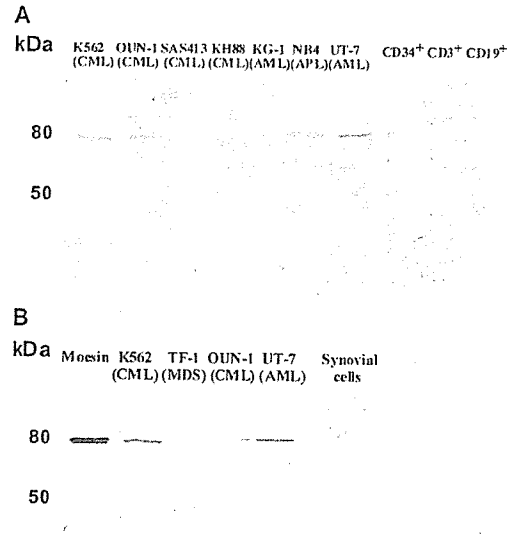


**Figure 4. Detection of moesin in the culture supernatant of myeloid leukemia cell lines, CD34+ cells, CD3+ T cells, CD19+ B cells, and synovial cells.** (A) Culture supernatants of various myeloid leukemia cell lines, CD34+ cells, CD3+ T cells, and CD19+ B cells were subjected to Western blotting using antimoesin Abs. (B) Exosomal fractions prepared from the culture supernatants of the 4 positive cell lines and synovial cells were also examined by Western blotting.

When the purified exosome fraction was examined by electron microscopy, the presence of small particles which were compatible in size with that of the exosomes was observed (data not shown).

### Relationship of antimoesin Abs with either other markers or the response to IST

We previously reported that Abs specific to DRS-1 are detectable in 38% of patients with PNH+ AA.[13] We then studied the relationship between the presence of antimoesin Abs with the presence of anti–DRS-1 Abs in 45 patients with AA. Antimoesin Abs were detectable in 68% of patients with anti–DRS-1 Ab+, whereas they were detectable in only 27% of patients with anti–DRS-1 Ab− ($P = .007$). Twenty-eight patients recently diagnosed underwent ATG + CsA or CsA therapy after examination of their blood for the presence of antimoesin Abs, anti–DRS-1 Abs, and small populations of PNH-type cells (Table 1). There were no significant differences in the rate of response to ATG + CsA or CsA between patients showing antimoesin Abs and those not showing antimoesin Abs (75% versus 67% for ATG + CsA; 100% versus 75% for CsA). When all 3 markers were assessed, 13 (76%) of 17 and 9 (100%) of 9 patients showing at least 1 of the 3 makers improved with ATG + CsA and CsA alone, respectively, whereas none of 2 patients not showing any of these markers responded.

## Discussion

The present study revealed that Abs specific to moesin are detectable in the serum of approximately 37% of patients with AA.

**Table 1. Relationships between the presence of PNH-type cells and auto-Abs in patients recently diagnosed with AA and clinical significance of antimoesin Abs**

| Patient | Age, y | Sex | Severity | PNH | Anti-DRS-1 Ab | Antimoesin Ab | Response to IST | IST |
|---|---|---|---|---|---|---|---|---|
| 1 | 25 | M | M | + | + | + | + | ATG + CsA |
| 2 | 29 | F | M | + | + | + | + | ATG + CsA |
| 3 | 60 | M | M | + | + | + | + | ATG + CsA |
| 4 | 64 | F | S | + | + | + | + | ATG + CsA |
| 5 | 66 | F | M | + | + | + | + | ATG + CsA |
| 6 | 73 | F | M | + | + | + | + | ATG + CsA |
| 7 | 49 | M | S | + | + | + | − | ATG + CsA |
| 8 | 66 | M | M | + | + | − | + | ATG + CsA |
| 9 | 17 | M | M | + | − | + | + | ATG + CsA |
| 10 | 73 | M | S | + | − | + | + | ATG + CsA |
| 11 | 72 | M | M | + | − | + | − | ATG + CsA |
| 12 | 22 | M | S | + | − | − | + | ATG + CsA |
| 13 | 62 | M | S | + | − | − | + | ATG + CsA |
| 14 | 83 | F | S | + | − | − | + | ATG + CsA |
| 15 | 23 | M | S | − | + | − | − | ATG + CsA |
| 16 | 63 | M | M | − | − | + | + | ATG + CsA |
| 17 | 77 | F | S | − | − | + | − | ATG + CsA |
| 18 | 28 | M | M | − | − | − | − | ATG + CsA |
| 19 | 34 | F | M | + | + | + | + | CsA |
| 20 | 42 | M | M | + | + | + | + | CsA |
| 21 | 74 | F | S | + | + | + | + | CsA |
| 22 | 78 | F | M | + | + | + | + | CsA |
| 23 | 70 | F | M | + | + | − | + | CsA |
| 24 | 75 | F | M | + | − | + | + | CsA |
| 25 | 47 | F | M | + | − | − | + | CsA |
| 26 | 79 | M | M | + | − | − | + | CsA |
| 27 | 43 | F | M | − | − | + | + | CsA |
| 28 | 39 | F | M | − | − | − | − | CsA |

AQ: 26 M indicates XXX; S, XXX; −, XXX.

Moesin is an intracellular protein that links the cell membrane and cytoskeleton and mediates the formation of microtubules and cell-adhesion sites as well as ruffling of the cell membrane.[25] This membrane-linking protein is expressed by various blood cells, including megakaryocytes and granulocytes, but its expression is localized inside the cell membrane and not on the cell surface. It is therefore unlikely for antimoesin Abs to affect the function and viability of hematopoietic cells. It is also unlikely that T cells specific for moesin play a role in the inhibition of hematopoietic stem cells in patients with AA because moesin is expressed by various kinds of cells other than blood cells.[26,27] Nevertheless, the presence of antimoesin Abs appears to reflect the immune pathophysiology of bone marrow failure because it correlates with the presence of small population of PNH-type cells which are well associated with the immune pathophysiology of AA.[8]

A previous study demonstrated the presence of antimoesin Abs in 14% of patients with rheumatoid arthritis.[23] None of the patients with antimoesin Abs$^+$ with rheumatoid arthritis in the previous report and of the 11 patients in our study showed cytopenia. In contrast, none of our patients with AA with antimoesin Abs showed symptoms characteristic of rheumatoid arthritis or laboratory findings such as positive rheumatoid factors. There was no difference in the specificity pattern of antimoesin Abs in patients with AA and rheumatoid arthritis. However, a case-control study on AA conducted by IAAS revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA,[28] and moesin was detectable in the exosomes derived from leukemia cell lines as well as from synovial cells of a patients with rheumatoid arthritis. It is therefore possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin.

In T-cell diseases such as AA, the presence of auto-Abs has not attracted much attention from hematologists for a long time. Recently, 3 different auto-Abs specific to kinectin,[29] DRS-1,[13] and anti-postmeiotic segregation increased 1[30] were detected in the serum of patients with AA. All of these Abs were identified through the immunoscreening of serum with a cDNA library derived from fetal liver cells or a leukemia cell line. The Abs to moesin are unique in that they were identified through their direct binding to protein in the denatured lysate of UT-7 cells. Moreover, moesin is the first autoantigen that was successfully identified in autoimmune diseases by using auto-Abs in patient's serum and peptide mass fingerprinting.

AQ: 22

Although the serologic identification of antigens by recombinant expression cloning (SEREX) is a useful assay for identifying novel antigens recognized by a small amount of auto-Abs in serum,[13,29] insignificant antigens are often identified because of the low specificity of the assay. Antigens detected by immunoblotting with serum may be significant when the assay revealed distinct bands. However, it is often difficult to identify the amino acid sequence of target antigens in cell lysates with peptide mass fingerprinting because of the presence of many proteins other than the target antigens which are eluted from the cut band. Moesin was successfully identified using the culture supernatant of UT-7 cells as a template because it was secreted from the cell line as a protein in the exosome and formed a single band in the polyacrylamide gel. Immunoblotting of exosomal fractions from culture supernatants followed by peptide mass fingerprinting thus appears to be a powerful method for identifying novel antigens which potentially elicit antibody production.

Our study showed that moesin was secreted into culture medium from various leukemia cell lines and synovial cells of a rheumatoid arthritis patient as an exosomal protein. Previous studies have shown that exosomes secreted from B lymphocytes and mesothelioma cells contain various intracellular proteins, including moesin,[19,24] and that some leukemia/lymphoma cell lines such as K562[21] and Daudi[22] secrete exosome-containing cytosolic proteins. However, moesin has not yet been detected in the exosomal fractions derived from cells from leukemia cell lines and synovial cells. Immunoblotting in the present study has shown the exosome fraction derived from the culture supernatants of the leukemia cell lines to contain moesin. Exosomes are known to be capable of presenting antigen by itself directly or indirectly through being ingested by antigen-presenting cells to helper T cells and eliciting specific Ab production.[31,32] It is conceivable that immature hematopoietic cells and B lymphocytes of patients with AA may also secrete a low amount of moesin as an exosome, leading to the induction of antimoesin Abs. The presence of moesin in the culture supernatant of CD34$^+$ cells and B cells from a healthy person supports this hypothesis.

The prevalence of high antimoesin Abs was significantly higher in patients with PNH$^+$ than in patients with PNH$^-$. A similar association was observed between anti–DRS-1 Ab and PNH-type cells in our previous study. In immune-mediated bone marrow failure such as PNH$^+$ AA, the immune system attack, particularly by CD4$^+$ T cells, is thought to damage hematopoietic stem cells. Various cytokines produced by CD4$^+$ T cells in the process of the immune attack may stimulate hematopoietic cells and B cells to secrete moesin, leading to a breakdown of immune tolerance toward moesin. Although the presence of antimoesin Abs was not associated with a good response to IST in our small series of patients, the detection of antimoesin Abs in combination with anti–DRS-1 Abs and PNH-type cells may help predict a good response to IST. Indeed, the rate of response to IST in 28 patients with AA showing at least 1 of the 3 makers was 85%, whereas none of the 2 patients with AA not showing any of the markers responded. The presence of such auto-Abs may particularly serve as a good marker for immune pathophysiology when sufficient numbers of blood cells for flow cytometry to detect PNH-type cells are not available because of severe bone marrow failure. The predictive value of these autoantibodies needs to be tested in a prospective clinical study.

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of Dokkyo Medical University; K. Shindo and M. Tashima of Nagahama City Hospital; A. Wakita of Nagoya City Higashi Hospital; N. Takahashi of Chichibu Daiichi Hospital; T. Tamaki of Izumisano City Hospital; F. Nakahara and S. Iki of NTT Kanto Hospital; K. Sugiura of Kyoritu Sogo Hospital; M. Iida, Y. Nakamura, and M. Yamaguchi of Ishikawa Prefectural Central Hospital; K. Koike of Asahikawa Red Cross Hospital; M. Morioka of Aiiku Hospital; K. Kanaya of Tokyo Medical University Hachioji Medical Center; T. Fukushima of Kanazawa Medical University Hospital; Y. Tamai of Hirosaki University Hospital; T. Oonaka of University of Occupational and Environmental Health; N. Hyakunan of Ryukyu University Hospital; K. Kyoda of Kouseiren Takaoka Hospital; K. Kumano of Hokkaido University Hospital; Y. Maesako of Tenri Hospital; K. Suzuki of Matsusaka Chuo General Hospital; H. Ishida and N. Uoshima of Matsushita Memorial Hospital; E. Ishii of Saga Medical University Hospital; N. Ichikawa of Nagano Red Cross Hospital; S. Yagima of Hamamatsu Medical Center; Y. Asano of Chihaya Hospital; H. Gondo of Hamanomachi Hospital.

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## Authorship

Contribution: H.T., S.N., and T.C. designed the research, analyzed data, and wrote the paper; M.Y. and S.I. performed electron microscopic examination; X.F. and X.L. performed research; K.O. performed peptide mass fingerprinting; C.S. analyzed data.

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EXPERIMENTAL HEMATOLOGY

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# Roles of DRB1*1501 and DRB1*1502 in the pathogenesis of aplastic anemia

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*Objective.* **Although a number of reports have documented a significantly increased incidence of HLA-DR15 in aplastic anemia (AA), the exact role of HLA-DR15 in the immune mechanisms of AA remains unclear. We herein clarify the difference between DRB1*1501 and DRB1*1502, the two DRB1 alleles that determine the presentation of HLA-DR15, in the pathophysiology of AA.**

*Materials and Methods.* **We investigated the relationships of the patients* HLA-DRB1 allele with both the presence of a small population of $CD55^{-}CD59^{-}$ (PNH-type) blood cells and the response to antithymocyte globulin (ATG) plus cyclosporin (CsA) therapy in 140 Japanese AA patients.**

*Results.* **Of the 30 different DRB1 alleles, only DRB1*1501 (33.6% vs 12.8%, $p_c < 0.01$) and DRB1*1502 (43.6% vs 24.4%, $p_c < 0.01$) displayed significantly higher frequencies among the AA patients than among a control. AA patients possessing HLA-DR15 tended to be old, and especially, the frequency of DRB1*1502 in patients 40 years of age and older (52.4%) was markedly higher than that in those younger than 40 years old (16.2%, $p_c < 0.01$). Only DRB1*1501 was significantly associated with the presence of a small population of PNH-type cells and it also showed a good response to ATG plus CsA therapy in a univariate analysis. A multivariate analysis showed only the presence of a small population of PNH-type cells to be a significant factor associated with a good response to the immunosuppressive therapy ($p < 0.01$).**

*Conclusions.* **Although both DRB1*1501 and DRB1*1502 contribute to the development of AA, the methods of contribution differ between the two alleles.** © **2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Although the etiology remains unclear, the immune destruction of hematopoietic stem cells has been considered the most important mechanism of bone marrow failure in AA [1]. One important finding supporting the role of such autoimmune mechanisms in AA is the high incidence of a certain HLA allele in AA patients. A number of reports have documented a significantly increased incidence of HLA-DR2 or the split antigen HLA-DR15 in AA [2–5]. We previously demonstrated a strong association between DRB1*1501 and a susceptibility to AA, in which the hematopoietic function improves with administration of cyclosporin A (CsA) [6]. Some reports have also demonstrated that HLA-DR15 or DRB1*1501 can predict the response to immunosuppressive therapy (IST) in patients with AA and myelodysplastic syndrome (MDS) [7–9], while others have failed to identify HLA-DR15 as a predictor for the response to antithymocyte globulin (ATG) therapy [3,10,11].

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