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ORIGINAL ARTICLE

Distinctive expression of myelomonocytic markers and down-regulation of CD34 in acute myelogenous leukaemia with FLT3 tandem duplication and nucleophosmin mutation

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Abstract

Objective: Patients with acute myelogenous leukaemia (AML) show co-existing frequently internal tandem duplications of FLT3 (FLT3-ITD) and mutations of nucleophosmin (NPM1-Mt). We investigated the biological and clinical significance of FLT3-ITD and/or NPM1-Mt in this context. *Methods:* We analysed 89 AML patients according to whether NPM1 and FLT3-ITD were single mutants, double mutants, or wild type for both. *Results:* FLT3-ITD was detected in 19 of 89 patients (21.3%), while NPM1-Mt was detected in 19 of 89 patients (21.3%); eight of 89 patients (9.0%) carried both FLT3-ITD and NPM1-Mt. By multivariate analysis, white blood cell count and peripheral blood blast cell count at diagnosis were significantly higher in patients with FLT3-ITD but not in those with only NPM1-Mt. NPM1-Mt was significantly related to female gender, normal karyotype, and M4 or M5 disease according to French–American–British criteria. In addition, leukaemic blast cells with NPM1-Mt, FLT3-ITD, or both expressed CD34 less frequently than wild-type blasts ($P < 0.0001$ and $P = 0.005$ respectively), while myelomonocytic markers such as CD11b and CD14 were expressed more frequently in patients with NPM1-Mt. *Conclusion:* FLT3-ITD may increase potential for cell proliferation to produce a leukaemic population; NPM1-Mt may cause cells to develop along the myelomonocytic lineage. Extensive analyses and detailed experiments will be required to clarify how NPM1 and FLT3 mutations interact in leukaemogenesis.

Key words acute myelogenous leukaemia; FLT3 internal tandem duplication; nucleophosmin mutation; myelomonocytic markers; CD34

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Acute myelogenous leukaemia (AML) is a heterogeneous haematologic malignant disease that can be distinguished by morphology, immunophenotyping or cytogenetic analyses. Approximately 50% of patients with AML carry chromosomal abnormalities that identify categories with significant clinical and prognostic features: patients with t(8;21), t(15;17) and inv(16) are considered to have 'good-risk' cytogenetics, while those with 5q-, 17q-, t(9:22), 11q23, and complex karyotypes are at greater risk for poor outcomes. On the other hand, conventional karyotypic analysis shows no chromosomal abnormalities in about half of patients with AML; while these patients with a normal karyotype have been considered an 'intermediate-risk' group (1), they are biologically and clinically the least well understood. Recent investigations have suggested involvement of several mutations of genes encoding transcription factors (AML1, CEBP/alpha) (2,3), receptor tyrosine kinases (FLT3, KIT) (4,5) and nucleophosmin (NPM1) (6), overexpression of the *BAALC* (brain and acute leukaemia, cytoplasmic) gene,

or overexpression of ETS-related gene in the pathogenesis of AML (7,8). One therefore needs to understand mechanisms by which these mutated genes are involved in leukaemogenesis and how then influence prognosis in AML.

FLT3, a member of the class 3 receptor tyrosine kinase family, is expressed on haematopoietic progenitor cells and is important for the survival and proliferation of early haematopoietic progenitors (9). An internal tandem duplication (ITD) of the FLT3 gene (FLT3-ITD) has been reported in 20–30% of adult patients with AML, especially in normal-karyotype and t(15;17)-positive patients (4,10,11). FLT3-ITD encodes an abnormal protein that can cause ligand-independent receptor dimerisation, autophosphorylation, and constitutive activation of downstream signalling pathways involved in cell proliferation, differentiation, and survival (12). Recent studies have shown that an activating FLT3 mutation is associated with higher white blood cell (WBC) counts and relapse rates, and may carry a worse prognosis than that for intermediate-risk AML patients in general (4,11,13). Accordingly, these patients can be assigned to a distinct subgroup to be considered for risk-adapted treatment options such as allogeneic and autologous stem cell transplantation (14).

Another novel mutation, the NPM1 gene mutation (NPM1-Mt), may be present in 25–45% of patients with AML (6,15–18). In normal haematopoiesis, wild-type nucleophosmin protein plays a key role in regulating protein synthesis, cell growth and cell proliferation, involving access to maturing ribosomes (19,20) and regulation of oncosuppressors such as ARF and p53 (6). In AML, the most common NPM1-Mt appears to be a four-base-pair insertion leading to a frameshift causing replacement of amino acids in the C-terminal portion of NPM1 (6). This mutation can result in loss of tryptophan residues 288 and 290 (or 290 only), and ultimately an aberrant cytoplasmic location of the protein product that may contribute to leukaemogenesis. NPM1-Mt has been reported to occur predominantly in patients with a normal karyotype, and to be associated with little or no CD34 expression and higher WBC and blast cell counts (18,21). Further, patients with NPM1-Mt harbour FLT3-ITD approximately twice as often as those with wild-type NPM1 (NPM1-Wt) (6,18). To investigate the consequences of FLT3-ITD and NPM1-Mt, we investigated pathologic and clinical features of AML subgroups with or without FLT3-Wt or NPM1-Wt among 89 AML cases. Multivariate analysis showed occurrence of double mutations three times more frequently than single mutations. A significant increase in WBC and blast cell counts was associated more closely with FLT3-ITD, while increased expression of monocytic markers and down-regulation of CD34 expression were noted in patients with NPM1-Mt. These results suggested that FLT3-ITD increased proliferation potential, while NPM1-Mt might favour leukaemic cell maturation along the myelomonocytic lineage. Together, these two mutations could bring about a leukaemia with specific myelomonocytic features.

Patients, materials and methods

Patients

We retrospectively analysed 89 AML patients with cell samples from the time of initial diagnosis up to December 2005 available for gene analyses. Patients with acute promyelocytic leukaemia, which very often is associated with FLT3-ITD (4,11), were excluded from the study. Patients studied included 56 men and 33 women, with a median age of 48 yr (Table 1). Chromosomal G-banding was carried out using a standard method. In each case, according to the International System for Human Cytogenetic Nomenclature guidelines, at least 20 mitotic events were analysed to exclude clonal abnormalities. Patient data including WBC and peripheral blood (PB) blast cell counts at the time of the initial diagnosis, morphologic types according to the French–American–British (FAB) classification and cytogenetic findings are shown in Table 1.

Patients were treated with a conventional remission-induction regimen consisting of idarubicin or daunorubicin for 3 d and cytosine arabinoside for 7 d. After achieving complete remission, patients were assigned to one of several types of postremission therapy including conventional consolidation chemotherapy, autologous PB stem cell transplantation (auto-PBSCT) and allogeneic haematopoietic stem cell transplantation (allo-SCT), based on prognostic factors such as chromosomal abnormalities. All patients or their guardians gave written informed consent in accordance with the requirements of the local Institutional Review Board.

Screening for FLT3-ITD and NPM1 mutations

Genomic DNA was isolated from bone marrow slides prepared at initial diagnosis, as described previously (14). Cells were dissociated from the slides, with contents then dissolved in phosphate-buffered saline. Genomic DNA was extracted using QIAamp DNA mini-kits (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations.

For FLT3-ITD, a polymerase chain reaction (PCR)-based amplification of genomic DNA was carried out as previously described (12), using primers 11F and 12R located in FLT3 exon 14 and 15. PCR products were separated by electrophoresis using a 3% Nusieve GTG agarose gel (Cambrex Bio Science Rockland, Rockland, ME, USA). FLT3-ITDs were detected as abnormally

Table 1 Clinical characteristics of the 89 acute myelogenous leukaemia patients according to FLT3-ITD/NPM1 mutational status

	FLT3-Wt/ NPM1-Wt	FLT3-Wt/ NPM1-Mt	FLT3-ITD/ NPM1-Wt	FLT3-ITD/ NPM1-Mt	Multivariate analysis (*P*-value)	
Number	59	11	11	8*	FLT3-ITD	NPM1-Mt
Female (%)	27.1	63.6	45.5	62.5	0.3492	0.0216
Age	46 (19-74)	49 (19-79)	52 (15-86)	51.5 (37-70)	0.6558	0.3736
FAB classification (%)						
M0	4 (6.8)	1 (9.1)	1 (9.1)	0		
M1	16 (27.1)	2 (18.2)	3 (27.3)	1 (12.5)		
M2	23 (39.0)	2 (18.2)	3 (27.3)	2 (25.0)		
M4	9 (15.3)	3 (27.3)	0	5 (62.5)		
M5	6 (10.2)	3 (27.3)	3 (27.3)	0		
M6	1 (1.7)	0	0	0		
M7	0	0	1 (9.1)	0		
M4 + M5	15 (25.4)	6 (54.5)	3 (27.3)	5 (62.5)		
others	44 (74.6)	5 (45.5)	8 (72.7)	3 (37.5)	0.7526	0.0159
Cytogenetics (%)						
Normal	25 (42.4)	9 (81.8)	8 (72.7)	7 (87.5)		
Favourable	9 (15.3)	0	0	0		
Intermediate	19 (32.2)	1 (9.1)	3 (27.3)	1 (12.5)		
Adverse	6 (10.2)	1 (9.1)	0	0		
Normal	25 (42.4)	9 (81.8)	8 (72.7)	7 (87.5)		
Others	34 (57.6)	2 (18.2)	3 (27.3)	1 (12.5)	0.0683	0.0109
WBC (10^9/L)	6.0 (1.2–362)	12.7 (1.4–92.1)	18 (4.1–200.4)	39.7 (1.9–177.3)	0.0006	0.4223
PB blast count (10^9/L)	1.64 (0–358.4)	5.8 (0–87.3)	15.6 (2.77–198.4)	33.5 (0.19–164.9)	0.0007	0.4369

*$P < 0.05$ by univariate analysis.

large products, which were cut out from the gel, purified with a QIAquick Gel Extraction kit (QIAGEN), and cloned into the PCR II-TOPO vector (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommendations. Sequencing was performed using an ABI PRISM BigDye terminator V 1.1 cycle sequencing kit and 21M13 and T7 with an ABI 310 Prism sequencer (Applied Biosystems, Foster City, CA, USA).

For the detection of NPM1 mutations, we amplified genomic DNA corresponding to exon 12 of NPM1 by PCR using the NPM1 primers: forward, 5′-CTA GAG TTA ACT CTC TGG TGG-3′ and reverse, 5′-CCT GGA CAA CAT TTA TCA AAC-3′. Amplified products were confirmed by electrophoresis and directly sequenced on an ABI 310 Prism sequencer using an ABI PRISM BigDye terminator cycle sequencing kit.

Immunophenotypic analysis

In all cases, immunophenotypic analyses were performed using bone marrow samples obtained at the time of diagnosis. Mononuclear cells (MNC) were separated by density-gradient centrifugation. The number of MNC collected was adjusted to 10^6 per tube. Selected monoclonal antibodies conjugated to fluorescein isothiocyanate, phycoerythrin and peridinin-chlorophyll protein were used at concentrations titrated to attain optimal staining. Immunophenotype measurements were performed with a multi-colour flow cytometer (FACScalibur or FACScanto, Becton Dickinson, San Jose, CA, USA). Leukaemic blasts were gated according to dim CD45 vs. low-side scatter and analysed further using various combinations of conjugated monoclonal antibodies: CD19/CD13/CD45, CD7/CD33/CD45, CD22/CD33/CD45, CD34/CD56/CD45, CD10/HLA-DR/CD45, CD15/CD11b/CD45, CD3/CD5/CD45, CD4/CD8/CD45, CD2/CD38/CD45, CD41/CD14/CD45, CD16/CD56/CD45, CD45/GPA, CD20/CD10/CD45 and a negative control for autofluorescence. All bone marrow samples contained over 20% blasts, and at least 20 000 events per tube were assessed. Each antigen was considered positive if expressed at a frequency exceeding 20% of cells gated as blasts. Data were analysed with FLOWJO software (TreeStar, Ashland, OR, USA).

Statistical analysis

For univariate comparisons, including cases with FLT3-Wt vs. those with FLT3-ITD; cases with NPM1-Wt vs. those with NPM1-Mt; and cases with FLT3-Wt/NPM1-Wt vs. those with FLT3-Wt/NPM1-Mt vs. those with FLT3-ITD/NPM1-Wt vs. those with FLT3-ITD/NPM1-Mt, we examined categorical variables such as gender, FAB classification, cytogenetic findings, response to induction therapy, type of postremission therapy and immunophenotype with the chi-squared test or Fisher's

exact test. Numerical variables such as age, WBC at diagnosis and PB blast cell count at diagnosis were compared with Student's *t*-tests or analysis of variance.

To evaluate effects of FLT3 and NPM1 with adjustment for one another, logistic regression analysis and multiple linear regression analysis were carried out using each categorical or numerical variable as a dependent variable, by designating both FLT3 and NPM1 as independent variables in the model.

Overall survival (OS) was measured from the date of diagnosis until the date of death. OS was calculated according to the Kaplan–Meier method, and comparisons were made with the log-rank test in the univariate analysis. Furthermore, an analysis of survival using Cox's proportional hazard model was conducted with FLT3-ITD and NPM1-Mt as independent variables. *P*-values <0.05 were considered to indicate statistical significance. All calculations were performed using BMDP statistical software.

Results

Incidence of FLT3-ITD and/or NPM1 mutations

FLT3-ITD was detected in 19 of 89 patients (21.3%). Although sequencing of the PCR product indicated that FLT3-ITD varied in both position and length from 21 to 102 bp, it was always in-frame and limited to the juxtamembrane domain. A representative result is shown in Fig. 1A. NPM1 mutations were detected in 19 of 89 patients analysed (21.3%). All mutations occurred at position 960 of the NPM coding sequence. Among the 19 patients with NPM1 mutations, mutation A, the most common type (6) with insertion of four bases, TCTG was detected in 16 (84.2%). The remaining three patients (15.8%) had mutation B, an insertion of CATG (Fig. 1B).

FLT3-ITD was present in eight of 19 patients with NPM1-Mt (42.1%), when compared with only 11 of 70 patients with NPM1-Wt (15.7%), ($P = 0.029$). Similarly, NPM1-Mt was detected in eight of 19 patients with FLT3-ITD (42.1%), but only 11 of 70 patients with FLT3-Wt (15.7%). These results indicate that NPM1 mutations and FLT3-ITD represent partially overlapping subgroups in AML. We therefore performed further analyses in terms of four groups: NPM1 and FLT3-ITD single mutants (FLT3-ITD/NPM1-Wt, $n = 11$ and FLT3-Wt/NPM1-Mt, $n = 11$); double mutants (FLT3-ITD/NPM1-Mt, $n = 8$); and subjects with wild-type alleles of both loci (FLT3-Wt/NPM1-Wt, $n = 59$).

Characteristics and outcome

Characteristics of patients in the four groups above are summarised in Table 1. Age distribution did not differ between groups. In agreement with some previous reports (18,21), we detected NPM1-Mt more frequently in female patients (12 of 33, 36.4%) than in men (seven of 56, 12.5%), ($P = 0.022$). By multivariate analysis, median WBC ($P = 0.0006$) and PB blast cell counts ($P = 0.0007$) at diagnosis were significantly higher in FLT3-ITD patients, while NPM1-Mt was not associated

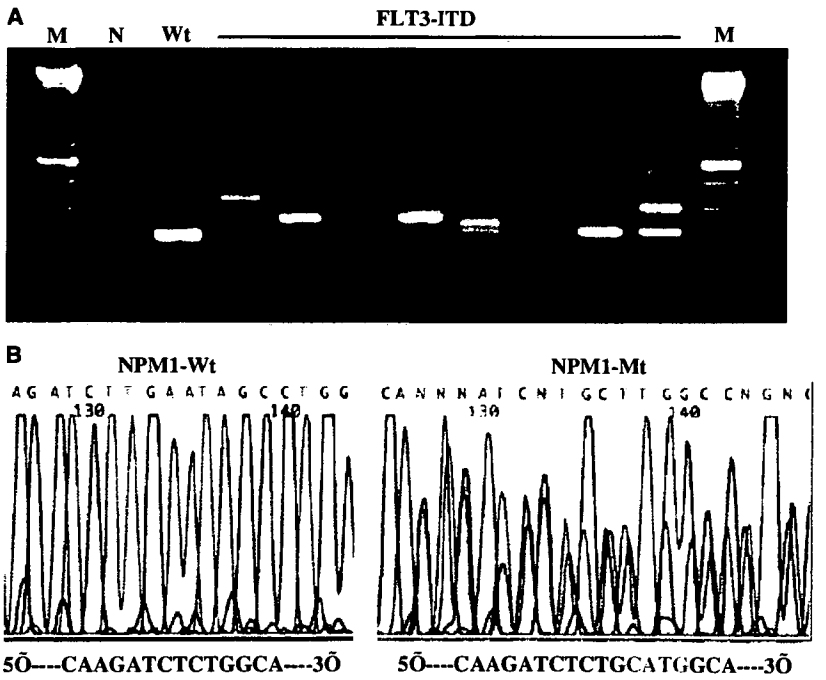

Figure 1 (A) PCR-based detection of FLT3-ITD in acute myelogenous leukaemia (AML). Single bands of 328 bp mark the wild-type FLT3 (Wt), but additional high molecular-weight bands of different sizes are found according to the length of duplicated fragments in AML with FLT3-ITD. M; 100 bp size marker, N; negative control (water). (B) Representative sequence results with and without NPM1 mutation. A 'Type-B' 4-bp insertion was detected at position 960 of the NPM1 gene in this case of mutated NPM1 (NPM1-Mt).

with high WBC or PB blast cell counts (P = 0.422 and 0.437 respectively). A significant difference also was seen in subtype of AML: NPM1-Mt was found predominantly in M4 and M5 (P = 0.016). In contrast, these subtypes did not differ in frequency of FLT3-ITD (P = 0.753).

We characterised cytogenetically all patients. As shown in Table 1, NPM1-Mt was associated with a normal karyotype (P = 0.011), and FLT3-ITD patients tended to have a normal karyotype (P = 0.068). No patient had NPM1-Mt or FLT-ITD in subgroups with t(8;21) or inv16.

All patients were treated with conventional remission induction-chemotherapy as described above. Complete remission rates were similar among between the four groups defined by mutation: 78.0% in FLT3-Wt/NPM1-Wt; 81.8% in FLT3-Wt/NPM1-Mt; 72.7% in FLT3-ITD/NPM1-Wt and 87.5% in FLT3-ITD/NPM1-Mt. Responders were treated further with various postremission therapies including consolidation chemotherapy, auto-PBSCT, and allo-SCT. OS for 5 yr was obtained in 41.65% of the FLT3-Wt/NPM1-Wt group; 64.65% of FLT3-Wt/NPM1-Mt; 44.55% of FLT3-ITD/NPM1-Wt; and 41.67% of FLT3-ITD/NPM1-Mt. Although no significant difference in OS was noted between groups, patients with FLT3-Wt/NPM1-Mt tended to have better outcomes as reported previously (16,18).

Immunophenotype

Expression of surface markers on leukaemic cells from patients in the four groups is shown in Table 2. Myeloid antigen (CD13 and CD33) and HLA-DR were detected in most cases, while T-lymphoid antigens (CD2 and CD3) and B-lymphoid antigens (CD10, CD19, and CD20) were detected in only a few cases. CD7, a pan-T-cell antigen, often is reported to be co-expressed on AML blasts; some investigators have suggested that CD7 expression on AML cells represents an unfavourable risk factor reflecting leukaemic transformation at an early stage of haematopoietic differentiation (22). Frequency of CD7 expression in our study did not differ significantly between the four groups. By multivariate analysis, myelomonocytic markers such as CD11b and CD14 were expressed more frequently on AML blasts in NPM1-Mt cases (P = 0.046 and 0.042 respectively). In addition, CD4, a marker frequently expressed at a low level in myelomonocytic leukaemias (23), was detected in 10 of 19 NPM1-Mt cases (P = 0.1079). These immunophenotypic results are compatible with a significantly high proportion of FAB subgroups M4 and M5 among NPM1-Mt cases (Table 1). Both FLT3-ITD (P = 0.005) and NPM1-Mt (P < 0.0001) were significantly associated with CD34 expression as an independent variable, with respective odds ratio of 5.7 (FLT3-ITD vs. FLT3-Wt) and 13.3 (NPM1-Mt vs. NPM1-Wt); the higher ratio indicated that NPM1 mutations had a closer relationship with low CD34 than did FLT3-ITD.

Discussion

Recent studies frequently have detected FLT3-ITD and NPM1-Mt in patients with normal-karyotype AML, with the two mutations often coinciding (6,18); accordingly,

Table 2 Surface marker profile of leukaemic blast cells in the 89 acute myelogenous leukaemia patients according to FLT3-ITD/NPM1 mutational status

	FLT3-Wt/ NPM1-Wt	FLT3-Wt/ NPM1-Mt	FLT3-ITD/ NPM1-Wt	FLT3-ITD/ NPM1-Mt	Multivariate analysis (*P*-value)	
Number	59	11	11	8	FLT3-ITD	NPM1-Mt
Antigen						
CD2	3 (5.1)	0	2 (18.2)	0	0.1709	0.0651
CD3	0	0	1 (9.1)	0	0.0523	0.2883
CD4	22 (37.3)	7 (63.6)	3 (27.3)	3 (37.5)	0.2292	0.1079
CD7	11 (18.6)	1 (9.1)	3 (27.3)	2 (25.0)	0.3041	0.4946
CD10	0	0	0	0		
CD11b	14 (23.7)	5 (45.5)	1 (9.1)	3 (37.5)	0.2779	0.046
CD13	57 (96.6)	9 (81.8)	8 (72.7)	8 (100)	0.5957	0.5957
CD14	6 (10.2)	3 (27.3)	1 (9.1)	3 (37.5)	0.7846	0.042
CD15	18 (30.5)	5 (45.5)	2 (18.2)	4 (50.0)	0.5893	0.1077
CD19	4 (6.8)	0	0	0	0.2355	0.2355
CD20	0	0	0	0		
CD33	56 (94.9)	11 (100)	9 (81.8)	8 (100)	0.1709	0.0651
CD34	47 (79.7)	3 (27.3)	5 (45.5)	0	0.0054	<0.0001
CD56	6 (10.2)	3 (27.3)	1 (9.1)	0	0.2208	0.3269
HLA-DR	56 (94.9)	8 (72.7)	8 (72.7)	6 (75.0)	0.1431	0.1431
GPA	1 (1.7)	0	0	0	0.5600	0.5600

they may be directly and co-operatively involved in the pathogenesis of AML. To more precisely identify factors associated with FLT3-ITD and/or NPM1-Mt, we performed multivariate as well as univariate analysis that included clinical and pathologic features. We detected NPM1 mutations in 21.3% and FLT3-ITD mutations in 21.3% of all AML patients analysed, a frequency essentially consistent with some previous reports (10,17,18). Double mutations were detected about three times more frequently than single mutations, with the frequency at which FLT3-ITD and NPM1-Mt occurred together with other mutated genes being about the same. In agreement with some previous univariate analyses (6,10), we observed a close association between normal karyotype and FLT3-ITD and/or NPM1-Mt by univariate analysis (FLT3-ITD vs. FLT3-Wt, $P = 0.0357$; NPM1-Mt vs. NPM1-Wt, $P = 0.0088$; comparing all subgroups, $P = 0.0081$). However, multivariate analysis disclosed that only NPM1-Mt was significantly associated with normal karyotype ($P = 0.0109$), while FLT3-ITD only tended to be associated ($P = 0.0683$); NPM1-Mt therefore showed a closer relationship. Similarly, some previous studies have shown that NPM1-Mt and FLT3-ITD each are associated with higher leucocyte and blast cell counts (15–18); while we also found significantly increased numbers of leucocytes and blast cells in patients with FLT3-ITD ($P = 0.0002$, $P = 0.0002$) and with double mutants ($P = 0.0019$, $P = 0.0018$); NPM1-Mt alone showed only a marginal relationship to higher numbers of leucocytes ($P = 0.0929$) and blast cells ($P = 0.0999$) by univariate analysis. In our multivariate analysis, a significant difference in leucocyte and blast counts was found only patients with FLT3-ITD ($P = 0.0006$, $P = 0.0007$), suggesting that FLT3-ITD mainly induces leukaemic cell proliferation. Recent researches have revealed molecular mutations play an increasing role for classification, prognostication and therapeutic strategies in AML (24,25). Despite our limitation of studying a small number of patients retrospectively, our NPM1-Mt patients showed a tend toward favourable clinical outcome, especially in patients without FLT3-ITD, in agreement with some recent investigations (16,18). More prospective studies will be required to establish an optimal method of treating AML patients with FLT3-ITD and/or NPM1-Mt.

Surface markers are a useful tool for assigning leukaemic blast cells to the myeloid or lymphoid lineage and for predicting which cells would be a target for leukaemic transformation during haematopoietic differentiation. Munoz *et al.* (26) demonstrated that AML cells with FLT3-ITD very often express myelomonocytic markers including CD11b, CD15, CD33 and CD36, and less commonly, immature markers such as CD34 and CD117. Similarly, evidence has been accumulating that antigen patterns in cases of NPM1-Mt correspond to a mature myeloid population with monocytic differentiation, showing lower expression of CD34 (6,15,18,21). In addition, M4 and M5 disease has been more frequent in FLT3-ITD (4,26) and in NPM1-Mt (16,17,21) cases. However, as these mutations often coincide with each other, which mutational event most affects the pathogenesis of AML with monocytic differentiation remains unclear. Although our univariate analysis did not reveal any significant difference in surface marker expression among the four subgroups except for CD34; multivariate analysis indicated that CD11b ($P = 0.046$) and CD14 ($P = 0.042$) were significantly associated with NPM1-Mt, independently of FLT3 status ($P = 0.2779$ and 0.7846 respectively). High expression of monocytic markers also was compatible with a more frequent finding of M4 and M5 disease in patients with NPM1-Mt ($P = 0.0159$), than in FLT3-ITD ($P = 0.7526$). Less frequent CD34 expression also was confirmed in both FLT3-ITD ($P = 0.0054$) and NPM1-Mt cases ($P < 0.0001$) independently of each other in the multivariate analyses. Less frequent CD34 expression was more closely associated with NPM1-Mt than FLT3-ITD (odds ratio, 13.3 vs. 5.7), which paralleled the more frequent diagnosis of mature myelomonocytic leukaemia, M4 and M5. Thus, our findings clearly indicated a close association between NPM1-Mt and monocytic features of AML.

Why FLT3 and NPM1 mutations often occur together remains unclear, as do which mutation precedes the other in the process of leukaemogenesis and how the two mutations function co-operatively. Multivariate analyses demonstrated distinctive pathologic and clinical features for each mutation. Significantly increased WBC and blast cell counts in FLT3-ITD may be associated with cell proliferation, while higher expression of monocytic markers and down-regulation of CD34 expression in NPM1-Mt may reflect differentiation toward the monocytic lineage. Recent findings suggest that FLT3 mutations occur in the primitive $CD34^+$ $CD38^-$ fractions, where a rare 'leukaemic stem cells' population might exist despite less frequent CD34 expression in AML with FLT3-ITD (27). Consequently, we would explain the pathogenesis of AML with FLT3-ITD and NPM1-Mt as follows: FLT3-ITD arises in the most primitive $CD34^+$ $CD38^-$ fractions and together with NPM1 mutations impairs differentiation of the accumulated blast cells, causing these cells to develop along the myelomonocytic lineage. Analyses of larger numbers of patients and detailed molecular investigations will be required to address how NPM1 and FLT3 mutations interact in leukaemogenesis to induce leukaemias specific to the myelomonocytic lineage.

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LETTER TO THE EDITOR

Marked improvement of cardiac function early after non-myeloablative BMT in a heavily transfused patient with severe aplastic anemia and heart failure

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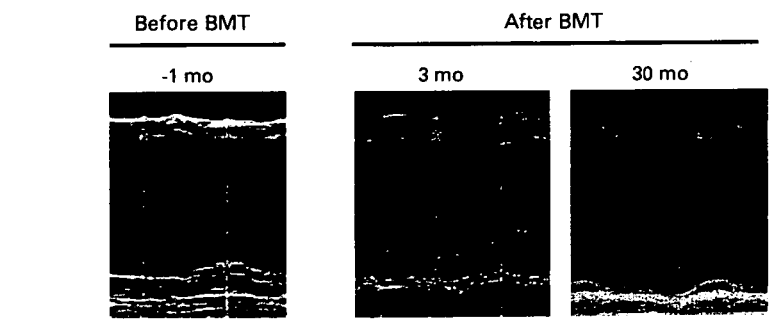
Allogeneic stem cell transplantation (allo-SCT) is one of the curative treatment options for patients with severe aplastic anemia (SAA). However, since multiple transfusions can cause organ damage such as heart failure, diabetes mellitus and liver dysfunction due to iron overload, the majority of heavily transfused patients with SAA would not tolerate the intensive conditioning regimens followed by allo-SCT. In addition, multiple transfusions also can sensitize the patients with alloantigens, resulting in the high risk of graft rejection. Non-myeloablative SCT (NST) has been developed to reduce regimen-related toxicities (RRT), especially for patients with poor organ function or advanced age, but it may be more frequently associated with graft rejection. Recently, several trials have utilized an NST regimen to reduce RRT and overcome graft rejection in the heavily transfused and allo-immunized SAA patients, using fludarabine in combination with melphalan[1,2] or cyclophosphamide.[3,4]

A 32-year-old man had suffered from SAA for 16 years. He had received several treatments with prednisolone, anabolic steroids, cyclosporine, ATG and G-CSF, without appreciable effects. The patient had no HLA-identical donors among his family or unrelated donor banks. Therefore, a large number of transfusions were required to keep him alive for 16 years. Owing to hemochromatosis secondary to the numerous RBC transfusions, he had developed cardiac failure, diabetes mellitus and hypothyroidism. An HLA-matched donor search had continued, and finally allo-BMT from an unrelated donor with HLA matched at A, B, C and DR loci was conducted in February 2004. On admission, the hemoglobin concentration was 6.0 g/dl; platelet count, 5.0×10^9/l; WBC, 1.6×10^9/l with 27% neutrophils. He presented with hepatosplenomegaly and congestive heart failure due to the hemochromatosis with a serum ferritin concentration of 11 506 ng/ml. Ultrasound cardiography (UCG) disclosed that the left ventricular EF (LVEF) was 20.9%, LV internal diameter in diastole (LVDd) 64.9 mm, LVD in systole (LVDs) 58.6 mm and LV wall motion was globally reduced, resembling a dilated cardiomyopathy (Figure 1). The serum concentration of brain natriuretic peptide (BNP) was increased up to 975 pg/ml (normal, <58.4 pg/ml). He was treated with metildigoxin, furosemide, spironolactone and losartan. The patient would not tolerate the standard conditioning regimen, including high-dose cyclophosphamide. Therefore, we decided to treat him using an NST conditioning regimen. He was given 180 mg/m^2 of fludarabine, 70 mg/m^2 of melphalan and 2 Gy of TBI, followed by transplantation of 3×10^8/kg bone marrow cells from an unrelated donor. Short-term methotrexate and tacrolimus were administered as prophylaxis against GVHD. Hematopoietic recovery was prompt and a chimeric analysis on day 23 disclosed that his bone marrow cells consisted entirely of donor-derived cells. Throughout conditioning, a low dose of



LVEF (%)	20.9	50.1	61.5
LVDs (mm)	58.6	39.7	32.9
LVDd (mm)	64.9	53.5	49.2

Figure 1 Ultrasound cardiography performed before and after allo-BMT. LVDd, LV internal diameter in diastole; LVDs, LV internal diameter in systole; LVEF, left ventricular (LV) ejection fraction.

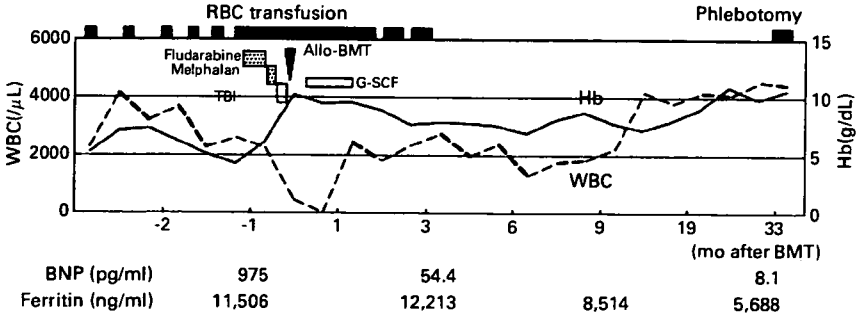


Figure 2 Clinical course of the patient after non-myeloablative BMT. BNP, serum level of brain natriuretic peptide (normal, <58.4 pg/ml).

dopamine and carperitide was administered continuously, and transfusions of RBC were performed to keep his hemoglobin concentration around 7 g/dl. Cardiac, renal and liver functions were well preserved, despite febrile events and administration of antibiotics following allo-BMT. According to Bearman's RRT scoring, the patient developed grade 3 mucositis but other organ RRT was scored below grade 2. On day 32, grade II acute GVHD developed, but was confined to the skin; this responded to prednisolone. Hematopoiesis gradually improved 3 months after allo-BMT; his WBC was 2.5×10^9/l and hemoglobin concentration 7.0 g/dl. Surprisingly, at this time point, his cardiac function had improved drastically. His LVEF had increased to 50.1% (Figure 1), and the serum BNP concentration returned to a normal range (54.4 pg/ml). He no longer manifested symptoms of heart failure, and required treatment with only losartan. However, his serum ferritin concentration was still high (12 213 ng/ml, Figure 2) and computed tomography (CT) revealed that iron deposition was still present in the heart as well as liver. Thirty months after allo-BMT, the WBC was 4.5×10^9/l and hemoglobin 12.5 g/dl without need for blood transfusion. The serum concentration of ferritin and BNP had decreased to 5688 ng/ml and 8.1 pg/ml, respectively (Figure 2). UCG showed a further significant functional improvement (Figure 1). This patient has been receiving phlebotomy to treat the iron overload for further improvement (Figure 2).

In this patient, the prompt improvement in cardiac function 3 months after BMT might not have correlated with the hematologic changes, since his hemoglobin concentration had not yet increased at that time, which had still required RBC transfusions. In addition, iron deposition still remained in the heart as seen in the CT scan. Therefore, the decrease in iron stores or amelioration of hemochromatosis by the successful BMT cannot account for the prompt improvement of left ventricular systolic function. It was recently reported from postmortem examinations that a fraction of cardiomyocytes from female patients who had undergone sex-mismatched allo-BMT were positive for Y chromosome staining, suggesting that donor-derived bone marrow cells can contribute to cardiomyocyte formation.[5] Animal experiments also have demonstrated that cardiac function improves several weeks after BMT, possibly through cell fusion or trans-differentiation, although the exact mechanisms remain to be determined.[6,7] Therefore, in this case, donor-derived bone marrow cells might partially contribute to the amelioration of cardiac dysfunction, rather than a decrease in iron deposition by the successful BMT resulted in an improvement in heart failure due to hemochromatosis. In this context, bone marrow cells as a stem cell source might be suitable for patients with organ damage, since bone marrow cells contain hematopoietic and mesenchymal stem cells, which can differentiate into other tissues such as cardiomyocytes to repair the injured organs.[6,8]

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INTERNAL MEDICINE

□ CASE REPORT □

Repeated Relapses of Acute Myelogenous Leukemia in the Isolated Extramedullary Sites Following Allogeneic Bone Marrow Transplantations

Yoshikane Kikushige, Ken Takase, Keiko Sata, Ken-ichi Aoki, Akihiko Numata, Toshihiro Miyamoto, Takahiro Fukuda, Hisashi Gondo, Mine Harada and Koji Nagafuji

Abstract

Isolated extramedullary (EM) relapses of acute myelogenous leukemia (AML) after allogeneic hematopoietic stem cell transplantation (allo-HSCT) have been reported to be rare, and are usually followed by bone marrow relapses. We report a 49-year-old man with AML with the unfavorable chromosome abnormality 7q-, who was treated by allo-HSCT. Fifteen months after allo-HSCT, the patient initially developed a relapse only in his inguinal lymph nodes, and then bone marrow relapse became evident one month after the EM relapse. Subsequently, the patient received chemotherapy and a second allo-HSCT from another donor, but he suffered another relapse in different EM sites including the skin and central nervous system with a persistently normal marrow. This case is characterized by repeated relapses in isolated EM sites after allo-HSCT and suggests that the anti-leukemic effects of chemotherapy and/or graft-versus-leukemia effects in the EM sites might not be so uniformly effective as that in the marrow. Accordingly, we should be aware that AML relapses can occur repeatedly only in isolated EM sites post allo-HSCT, resulting in treatment failure and a poor prognosis.

Key words: extramedullary, relapse, AML, allo-BMT, GVL

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Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for patients with acute myelogenous leukemia (AML) based upon graft-versus-leukemia (GVL) effects in addition to the intensive conditioning chemo-radiotherapy. However, some patients eventually develop a relapse following allo-HSCT, resulting in treatment failure and a poor prognosis. AML relapse usually occurs in the bone marrow, but a small fraction of patients develop extramedullary (EM) relapses either alone or concomitant with bone marrow relapse (1-5). Bekassy et al reported isolated EM relapse occurred after allo-BMT in 20 out of 3,071 AML patients (0.65%) (1). Little is known concerning the mechanism of EM relapse; however, the prognosis of patients with an EM relapse of AML is generally considered to be less favorable than that of AML patients with bone marrow relapse only (1, 6, 7).

We present a case of AML who developed a relapse confined to his inguinal lymph nodes 15 months after allogeneic bone marrow transplantation (allo-BMT). Following a second allo-BMT from another donor, the patient had multiple relapses in different EM sites such as the skin and central nervous system, with a persistently normal marrow. Frequent relapses in EM sites suggest that the GVL effect in the EM sites was not as potent as that in the bone marrow, where it remained effective. Thus, we should note that AML relapses can occur in isolated EM sites because the GVL effect might not be uniformly effective throughout the body following allo-HSCT.

Case Presentation

In June 2002, a 49-year-old Japanese man was referred to us for evaluation of leukocytosis and anemia. At the time of

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hospitalization, hemoglobin was 7.4 g/dl; platelet count, 135×10^9/l; white blood cell count, 32.3×10^9/l with 2% neutrophils, 9% lymphocytes, and 89% myeloblasts that stained positive for myeloperoxidase (MPO). A bone marrow aspirate was hypercellular with 91% myeloblasts, which were positive for CD7, CD13, CD15, CD33, CD34 and HLA-DR. Cytogenetic analysis of the bone marrow cells revealed 46 XY, 7q- in all metaphase cells. A diagnosis of AML-M1 type was made according to the French-American-British classification, and this patient with the 7q- chromosome abnormality was categorized as having a poor prognosis with an approximately 75% chance of relapse (8, 9). Extramedullary leukemia was not documented at diagnosis. The patient was treated with a conventional induction regimen consisting of idarubicin and cytarabine (CA), and he achieved a complete remission (CR). He was treated with two further cycles of consolidation chemotherapy consisting of intermediate-dose CA combined with mitoxantrone in the first course and etoposide in the second course, as described previously (10). During the consolidation chemotherapy, the patient developed invasive pulmonary aspergillosis, which was treated by administration of Amphotericin-B. Finally, local lung resection of left upper lobe was performed prior to allo-BMT.

In February 2003, the patient underwent an allo-BMT from an HLA matched unrelated donor after receiving busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg). Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine (CyA) and a short course of methotrexate (MTX). During the neutropenic period, the patient developed pulmonary abscess caused by Streptococcus pneumoniae, however, it gradually stabilized in response to antibiotic therapy. Engraftment was obtained on day 17, and a bone marrow examination demonstrated continuing CR. A chimeric analysis of the minisatellite variable number of tandem repeats disclosed that his bone marrow cells consisted entirely of donor-derived cells. On day 38, the patient developed grade I acute GVHD confined to his skin, which disappeared without any treatment. Four months after allo-BMT, the patient disclosed typical oral manifestations of chronic GVHD, which were resolved after adjusting the dose of CyA.

In May 2004, 15 months after BMT, the patient developed bilateral inguinal lymphadenopathy. He was otherwise asymptomatic with no organomegaly and a normal complete blood count. A biopsy of a left inguinal lymph node revealed the infiltration of medium-sized blastic cells with moderately irregular nuclei (Fig. 1). These cells were positive for CD7, CD13, CD33, CD34 and HLA-DR, the same phenotype as that seen in his bone marrow at presentation. Chimeric analysis demonstrated that the lymph node cells were recipient derived, and a deletion of 7q was documented by cytogenetic analysis. In contrast, a bone marrow aspirate showed no infiltration by AML cells and also a donor-derived pattern by chimeric analysis. Further systemic investigation including computed tomography of the whole body and lumbar puncture of his cerebrospinal fluid showed no leukemic involvement of his other organs except for the inguinal lymph nodes. Based on these observations, isolated EM relapse of AML confined to the lymph nodes was diagnosed. Another donor search was initiated immediately for a second allo-SCT. The immunosuppressant was discontinued to induce a GVL effect; however, no improvement in his lymphadenopathy was seen. One month after the discontinuation of immunosuppression, a bone marrow aspirate showed an increase in the number of AML blasts, which accounted for up to 11% of the bone marrow nucleated cells. Cytogenetic analysis also demonstrated the emergence of cells with a deletion of 7q, and 15% of his bone marrow cells were recipient-derived by chimeric analysis. Thus, the patient suffered a relapse of AML in his bone marrow following an isolated EM relapse in his lymph nodes. Re-induction chemotherapy consisting of daunorubicin and CA was administered. The inguinal lymphadenopathy disappeared soon after chemotherapy; however, the number of AML blasts still increased peripherally and in his marrow. Another two courses of chemotherapy, using the CAG-regimen (11), were ineffective in inducing a CR.

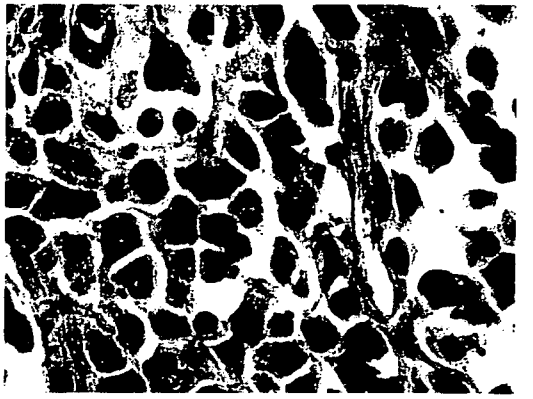

Figure 1. Left inguinal lymph node biopsy specimen showing diffuse infiltration of myeloid leukemic cells. Original magnification was × 1,000 (Hematoxylin & eosin stain).

In November 2004, the patient underwent a second allo-BMT from another HLA matched unrelated donor after receiving a reduced intensified conditioning (RIC) regimen consisting of fludarabine (180 mg/m^2), busulfan (8 mg/kg), and total body irradiation (2 Gy). Acute GVHD prophylaxis consisted of tacrolimus and a short course of MTX. Engraftment was confirmed on day 21, and the patient obtained and continued in CR without GVHD.

On day 150 after the second allo-BMT, the patient presented with multiple raised red-brown nodules, widely scattered over his trunk. A biopsy of a skin lesion showed medium sized blast cells, positive for MPO, CD34 and CD45, which were consistent with AML infiltrates in the skin (Fig. 2). The bone marrow aspirate remained normocellular, with normal maturation of all three lineages and the second donor-derived pattern. CT scans of the whole body showed

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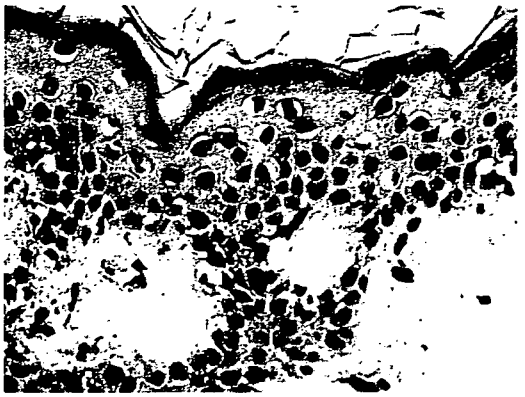

Figure 2. Biopsy specimen of skin lesions on chest demonstrating infiltration of leukemia cells. Original magnification was × 400 (Hematoxylin & eosin stain).

no lymphadenopathy or organomegaly, suggesting no evidence of leukemic infiltration into other sites. Thus, his relapse was documented in a different EM site after the second allo-BMT. Tacrolimus was discontinued to induce a GVL effect; however, his skin lesions expanded rapidly without developing any signs of GVHD. Thereafter, the patient developed a left facial nerve paralysis. His cerebrospinal fluid was infiltrated with AML cells despite repeated intrathecal injections of CA and MTX for prophylaxis of central nervous system (CNS) leukemia prior to allo-BMT. The patient died shortly after the documentation of CNS involvement.

Discussion

AML eventually relapses in 20% to 50% of AML patients after allo-HSCT. AML relapse following allo-HSCT usually occurs in the bone marrow where the leukemic burden is the heaviest, but isolated EM relapse can be detected rarely (1). Isolated EM relapse is usually accompanied by bone marrow relapse within 1 year, and is known to be a very poor prognostic factor, compared to that of medullary relapse after allo-HSCT (1, 6, 12). Thus, isolated EM relapse in AML is an increasingly recognized cause of treatment failure after allo-HSCT.

Predisposing factors that may contribute to the EM relapse of AML after allo-HSCT have been suggested. One factor may be associated with the intrinsic properties of the leukemic cells such as neural adhesion molecule NCAM (CD56) expression, chromosomal aberrations, which include t(8; 21), inv (16), and MLL rearrangement, and M4 and M5 by the FAB classification (1, 7). The present patient with AML-M1 did not have these risk factors for EM disease, and did not exhibit EM involvement at the initial presentation of relapse. The surviving leukemic cells might have transformed after the second round intensive chemotherapy followed by allo-BMTs, and have acquired the ability to adhere to dermal fibroblasts, facilitating their binding to EM tissues other than the bone marrow stroma, leading to the multiple isolated EM relapses (7, 13).

As another possible explanation, the EM sites might serve as sanctuary sites for the dormant leukemic clone after allo-HSCT, since the effect of anti-cancer drugs and/or immune cells and cytokines can be diminished in the EM sites due to the presence of a barrier (2, 7). Thus, following allo-HSCT, when the leukemic cells are still sensitive to the GVL effect, they may be suppressed in the marrow, but may escape from the immunosurveillance in the EM sites where the GVL may be less uniformly effective.

There is no established treatment strategy for EM relapse post allo-HSCT but experience has shown that the vast majority of patients with isolated EM relapse subsequently develop marrow disease and have a poor prognosis and rapidly succumb to their disease (1, 6, 7). This indicates that a small fraction of leukemic cells might be present in the bone marrow even in those patients in whom extramedullary disease appears to be isolated. To eliminate the residual leukemic cells resistant to prior therapies throughout the entire body, an aggressive treatment with chemotherapy plus a second allo-HSCT from another donor should be considered in some selected cases, especially in younger patients. However, the treatment of EM relapse post allo-HSCT is extremely difficult because of the cumulative toxicities of the previous high-dose chemo-radiotherapy and immunosuppression caused by GVHD and/or immunosuppressants administered. Most patients, including even the younger ones, cannot tolerate aggressive systemic chemotherapy and conventional conditioning regimen followed by a second allo-HSCT (1, 14). Localized therapy such as irradiation to the EM lesions cannot manage leukemic relapse, and these patients usually develop overt leukemia. Patients who have not suffered from GVHD may be offered discontinuation of immunosuppression and donor lymphocyte infusion (DLI) therapy to augment the GVL effects; however, data on the efficacy of DLI in patients with isolated EM relapse post allo-HSCT has not yet shown encouraging results (7, 15). Furthermore, the patient had histories of invasive pulmonary aspergillosis and pulmonary abscess. Based on these observations, in this case we performed a second allo-BMT with the RIC regimen from another donor at the documentation of EM relapse following the first allo-BMT to gain another GVL effect different from that of the first donor. Unfortunately, 5 months later he again developed isolated EM relapse confined to the skin and CNS without marrow disease. In this case, the AML relapse occurred in EM sites that might be inaccessible to chemotherapy and/or the GVL effect, while a full hematopoiesis from the donor was retained in his marrow where the GVL effect could have functioned well after the second allo-BMT. In addition, the prolonged immunosuppression by the double allo-BMT might impair the immunosurveillance against the residual leukemic cells. Thus, physicians should note that AML relapse can occur in isolated EM sites because the GVL effect might not be uniformly effective throughout the body and the surviving leu-

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kemic cells might acquire affinity to the EM sites as a consequence of transformation following allo-HSCT.

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ORIGINAL ARTICLE

Rituximab does not compromise the mobilization and engraftment of autologous peripheral blood stem cells in diffuse-large B-cell lymphoma

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To investigate effects of the preautografting administration of rituximab on the mobilization and engraftment of peripheral blood stem cells (PBSC), we retrospectively analyzed the outcomes of 43 newly diagnosed diffuse-large B-cell lymphoma patients who received CHOP chemotherapy with or without rituximab as a first-line treatment before autologous PBSC transplantation (PBSCT). There was no difference in the number of CD34$^+$ cells among PBSC between the non-rituximab and the rituximab groups. Although B-cells were completely depleted from PBSC in the rituximab group, we found no difference in the expression of CXCR-4, VLA-4 and c-Kit on PBSC, indicating that rituximab did not affect the expression of these adhesion molecules, which might be involved in the mechanism of mobilization. There was no significant difference in the recovery of neutrophils and platelets, transplant-related toxicity and post-transplant complications between the two groups. Despite the short follow-up, there was no significant difference in progression-free survival between the two groups. These results indicated no adverse effect of rituximab on the mobilization and engraftment of PBSC. Larger studies are required to determine the impact of rituximab on the mobilization and function of PBSC as well as whether a survival advantage exists in patients who undergo auto-PBSCT with rituximab.

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Introduction

The emergence of new, more effective therapies has benefited patients with aggressive non-Hodgkin's lymphoma (NHL). As high-dose chemotherapy with autologous stem cell rescue has been shown to provide a survival advantage over salvage chemotherapy in relapsed patients with NHL,[1,2] many investigators have attempted to extend the use of auto-SCT approaches for the treatment of aggressive NHL. Recent reports have revealed that high-dose chemotherapy followed by autologous peripheral blood stem cell transplantation (auto-PBSCT) is superior to standard chemotherapy as a primary treatment for newly diagnosed patients with aggressive NHL.[3,4] Moreover, rituximab has changed the treatment paradigm of CD20-positive lymphoma, and has improved response and survival rates in combination with chemotherapy,[5–7] and rituximab-containing chemotherapy is increasingly becoming the primary standard for patients with diffuse-large B-cell lymphoma (DLBCL). Recent trials have focused on how to incorporate rituximab into high-dose chemotherapy followed by auto-PBSCT as a first-line treatment,[8,9] including the concept of *in vivo* purging of lymphoma cells from the circulation before the collection of auto-PBSC.[10,11] However, there is little evidence to support an effect of rituximab on the mobilization and engraftment of auto-PBSC,[12,13] though rituximab might be associated with a poor mobilization and impaired engraftment of PBSC. In an attempt to clarify this issue, we retrospectively compared characteristics of collection and transplantation of auto-PBSC in DLBCL patients treated with a protocol consisting of six courses of the CHOP regimen and high-dose conditioning chemotherapy followed by auto-PBSCT with or without rituximab as a primary treatment. In addition, we also tested the expression of adhesion molecules such as VLA-4, CXCR-4, and c-Kit on mobilized PBSC in the two groups to elucidate whether rituximab affects the expression of these molecules, as degradation of adhesion molecules could lead to the release of stem/progenitor cells from bone marrow into peripheral blood.[14,15]

Patients and methods

Patient

Patients aged 15–65 years with newly and histologically diagnosed DLBCL, were enrolled in this study. CD20 expression was determined at each participating institution, and further immunostaining was performed on central review if the lineage assignment was ambiguous. Eligible patients had an ECOG performance status of 0–3, high-intermediate to high risk according to the International Prognostic Index,[16] and at least one objective, measurable disease parameter. Exclusion criteria included transformed follicular lymphoma, central nervous system involvement, inadequate organ function, concomitant malignancy and an active viral infection such as hepatitis B, hepatitis C and human immunodeficiency virus. This study was conducted in accordance with the ethical guidelines mandated by the Declaration of Helsinki. All patients signed informed consent forms approved by the institutional review board at each participating hospital.

Between May 2001 and January 2006, a total of 43 patients were enrolled into this retrospective analysis: 20 patients were treated with CHOP chemotherapy and autologous PBSCT from May 2001 to May 2003 before the era of rituximab treatment (non-rituximab group). After September 2003 when rituximab was approved for the treatment of DLBCL in Japan, 23 patients enrolled in our study were treated with exactly the same regimen mentioned above plus rituximab (rituximab group) as a first option. Patient characteristics were well balanced and not statistically different between the two groups with regard to sex, age and clinical conditions (Table 1).

Treatment

All patients were treated with a standard CHOP regimen consisting of cyclophosphamide 750 mg/m^2 on day 1, doxorubicin 50 mg/m^2 on day 1, vincristine 1.4 mg/m^2 to a maximum of 2 mg on day 1 and prednisone 100 mg/m^2 on days 1 through 5, every 21 days for six courses. Patients in complete remission (CR), CR of undetermined significance (CRu) or partial remission (PR) after three cycles of CHOP received high dose of etoposide 500 mg/m^2 for 3 days followed by granulocyte colony-stimulating factor (G-CSF) to mobilize PBSC as described previously.[17] PBSC were collected using a COBE Spectra (Gambro JAPAN Inc., Tokyo, Japan) blood cell separator.[17] The target cell dose was 2×10^6 CD34$^+$ cells/kg. Harvested PBSC were cryopreserved until used as described previously.[18]

After the collection of PBSC, all patients received additional three courses of CHOP. Thereafter, the patients in CR, CRu or PR underwent autologous PBSCT: the conditioning regimen consisted of ranimustine 200 mg/m^2 on day −8 and day −3, carboplatin 300 mg/m^2 on day −7 through −4, etoposide 500 mg/m^2 on day −6 through −4 and cyclophosphamide 50 mg/kg on day −3 and day −2. On day 0, unpurged PBSC were reinfused followed by administration of G-CSF 5 μg/kg. Engraftment was confirmed by a granulocyte count $>0.5 \times 10^9$/l and platelet counts $>20 \times 10^9$/l or independence of platelet transfusion.

On the other hand, 23 patients in the rituximab group received rituximab (Chugai Pharmaceutical Co., Tokyo, Japan) 375 mg/m^2 one day before the 2nd, 3rd, 5th and 6th CHOP regimen, on day −9 and day +1 after autologous PBSCT. Rituximab was also administered one day before the high-dose etoposide regimen, and one day before PBSC collection again for *in vivo* purging of circulating lymphoma cells to avoid contamination from lymphoma cells in the PBSC harvest. In total, patients in the rituximab group received eight courses of rituximab 3000 mg/m^2 in this protocol.

Table 1 Patient and disease characteristics

Characteristics	*R-patients (n = 23)*	*Non-R patients (n = 20)*	P
Age, median (range)	58 (21–65)	52 (15–65)	0.61
Male/female	10/13	12/8	0.87
IPI at diagnosis			
Intermediate-high	17	16	0.39
High	6	4	
Disease status at PBSCT			
CR	13	9	0.37
Cru	7	8	
PR	3	3	
Time in months to auto-PBSCT, median (range)	5.8 (4.4–8.5)	5.6 (4.6–9.8)	0.43

Abbreviations: IPI = international prognostic index; NS = not significant ($P > 0.05$); R = rituximab.

Cell staining and fluorescence activated cell sorter (FACS) analysis

Peripheral blood mononuclear cells (PBMNC) were prepared by thawing the frozen PBSC harvest samples, which were stored at −80°C.[18] PBMNC were stained with a Cy5-PE-conjugated lineage (Lin) cocktail (anti-CD3, CD4, CD7, CD8, CD11b, CD16, CD56 and glycophorin A; Caltag, Burlingame, CA, USA), fluorescein isothiocyanate-conjugated anti-CD19 (Becton Dickinson (BD) Pharmingen, San Jose, CA, USA), PE-conjugated anti-CXCR-4, VLA-4 and c-Kit (BD Pharmingen), allophycocyanin-conjugated anti-CD34 (BD Pharmingen) and PE-Cy7-conjugated anti-CD38 (Caltag) antibodies. Nonviable cells were excluded by propidium iodide staining. Expression of adhesion molecules was detected on progenitors using a highly modified triple laser (488 nm argon laser, 633 nm helium-neon laser and 407 nm crypton laser) FACS (FACS Aria; BD) as described previously.[15]

Statistical analysis

The test of independence between the rituximab and non-rituximab groups was made with the χ^2 test, Fisher's exact test, or the Kruskal–Wallis test, where appropriate. Distribution of time to progression-free survival (PFS) was summarized with Kaplan–Meier product limit estimators and compared by log-rank test.

Table 2 Peripheral blood stem cell mobilization characteristics

Characteristics	*R-patients (n = 23)*	*Non-R patients (n = 20)*	P
Duration from HD-VP to collection of PBSC, median days (range)	20 (16–22)	20 (15–21)	0.36
WBC count at PBSC collection, mean (range) ($\times 10^9$/l)	9.49±4.17 (4.30–18.26)	8.72±4.51 (3.70–19.30)	0.55
Neutrophil count at PBSC collection, mean (range) ($\times 10^9$/l)	7.07±4.14 (1.70–16.25)	6.33±4.29 (1.67–16.87)	0.44
Platelet count at PBSC collection, mean (range) ($\times 10^9$/l)	168.1±85.2 (49.3–373.0)	188.7±97.4 (37.3–282.7)	0.29
$CD34^+$ cell dose collected in PBSC ($\times 10^6$/kg), mean (range)	11.10±8.04 (2.23–27.9)	13.00±10.30 (6.35–51.00)	0.64
Percent of $CD34^+$ cells per PBSC MNC, mean (range)	3.66±3.27 (0.52–16.1)	3.98±2.09 (1.52–10.71)	0.85
Percent of $CD19^+$ B-cells per PBSC MNC, mean (range)[a]	0.06±0.05 (0.013–0.150)	0.89±0.39 (0.50–1.46)	<0.005

Abbreviations: R = rituximab; HD-VP = high-dose etoposide for PBSC mobilization; NS = not significant ($P > 0.05$).
[a]Content of $CD19^+$ B cells was analyzed in seven R-patients and seven non-R patients, respectively.

Table 3 Autologous PBSCT outcomes

Characteristics	*R-patients (n = 23)*	*Non-R patients (n = 20)*	P
$CD34^+$ cell dose reinfused in PBSC ($\times 10^6$/kg), median (range)	9.82±6.08 (2.28–25.85)	10.99±5.62 (3.41–25.50)	0.46
Time in days to neutrophils ($> 0.5 \times 10^9$/l), median (range)	9 (8–12)	9 (8–11)	0.39
Time in days to platelets ($> 20 \times 10^9$/l), median (range)	10 (8–15)	10 (8–14)	0.57
Time in days to platelets ($> 50 \times 10^9$/l), median (range)	12 (10–17)	13 (11–16)	0.8
Time in days to dependency of platelet transfusion, median (range)	8 (6–13)	9 (7–11)	0.15
Rate of > grade III of transplant-related toxicity[a]	17%	15%	0.45
Rate of any documented infection[b]	26%	30%	0.42
1-year PFS	80.20%	78.30%	0.59

Abbreviations: R = rituximab; NS = not significant ($P > 0.05$); PFS = progression-free survival.
[a]Transplant-related toxicities were scored using National Cancer Institute Common Toxicity Criteria.
[b]Any documented infection = any positive culture.

Results

Stem cell mobilization

One course of apheresis for the collection of PBSC was sufficient to obtain the number of $CD34^+$ cells needed for transplantation in all patients of both groups. There was no significant difference in white blood cell count (WBC) at collection (R vs NR group, mean WBC 9.49×10^9/l vs 8.72×10^9/l, $P = 0.55$), platelet counts at collection (mean, 168.1 vs 188.7×10^9/l, $P = 0.29$), duration from administration of etoposide to collection of PBSC (median day, 20 vs 20, $P = 0.36$), percentage of $CD34^+$ cells at harvest (mean, 3.66 vs 3.98%, $P = 0.85$), or $CD34^+$ cell dose collected (mean, 11.10 vs 13.00×10^6/kg, $P = 0.64$) between the two groups (Table 2).

Engraftment of PBSC

The characteristics of auto-PBSCT are shown in Table 3. Engraftment was rapid and documented in all patients. There was no significant difference in the $CD34^+$ cell dose reinfused (R vs NR group, mean $CD34^+$ cell dose 9.82 vs 10.99×10^6/kg, $P = 0.46$), days taken to achieve a granulocyte count of 0.5×10^9/l (median day, 9 vs 9, $P = 0.39$), and a platelet count of 20×10^9/l in platelet counts (median day, 10 vs 10, $P = 0.57$), or independence of platelet transfusion (median day, 8 vs 9, $P = 0.15$) (Table 3). There were no treatment-related deaths, and no significant difference in the frequency of more than grade 3 adverse events scored using the National Cancer Institute Common Toxicity Criteria between two groups (17 vs 15%, $P = 0.45$). Neutropenic fever occurred in the majority of patients in both groups; however, we found no difference in the prevalence of documented infections (26 vs 30%, $P = 0.42$).

Survival

The median follow-up period was 51 months in the non-rituximab group and 31 months in the rituximab group. At the time of reporting, there was no significant difference in 1-year PFS (R vs NR groups, 80.2 vs 78.3%, $P = 0.59$) (Table 3).

Expression of adhesion molecules on PBSC

Four courses of rituximab were administered before the collection of PBSC to eliminate DLBCL cells. Therefore, we compared the contents of B-cells in the harvest products to evaluate the efficacy of B-cell purging by rituximab. PBSC harvests contained a small fraction of $CD19^+$ mature-B cells (0.89±0.39% of MNC, $n = 7$) in the non-rituximab group, whereas only a few B cells were circulating (0.06±0.05% of MNC, $n = 7$) in the rituximab group ($P < 0.001$) (Table 2).

We next tested for the expression of c-Kit and adhesion molecules such as VLA-4 and CXCR-4 on immature $CD34^+$ stem/progenitor cells in the PBSC harvest, as downregulation of these molecules resulted in release of stem/progenitor cells from marrow and mobilization into the circulation.[15] Figure 1 shows the mean fluorescence intensity (MFI) for these molecules in seven patients in the non-rituxiamb group and seven in the rituximab group. There was no significant difference in MFI of c-Kit (NR vs R groups, 206.4±35.9 vs 182.0±22.0, $P = 0.15$), CXCR-4

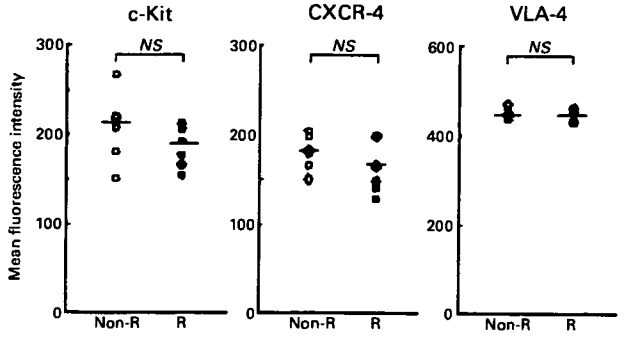

Figure 1 Comparative expression of c-Kit, CXCR-4 and VLA-4 on $CD34^+$ immature stem/progenitors in auto-PBSC harvests among the non-rituximab group (Non-R, open circle) and rituximab group (R, closed circle). Circles indicate median MFI for these molecules. NS, not significant.

(176.6 ± 17.9 vs 161.5 ± 27.2, $P=0.25$) and VLA-4 (457.7 ± 18.0 vs 454.0 ± 16.9, $P=0.69$) between the two groups.

Discussion

Promising results have been obtained for an initial treatment for NHL patients with high-dose chemotherapy followed by auto-PBSCT[3] as well as the addition of rituximab to standard chemotherapy.[6,7] Emerging new treatment strategies have focused on a combination of rituximab and high-dose chemotherapy with auto-PBSCT. Several trials have been conducted to design the optimal timing of rituximab administration during the treatment course of chemotherapy and auto-PBSCT.[9] A large number of protocols have incorporated rituximab administration preceding chemotherapy and pretransplant conditioning to enhance the chemosensitivity of lymphoma cells by exposure to rituximab.[19] In addition, to reduce or eliminate circulating lymphoma cells contaminating the PBSC harvest, concurrent administration of rituximab with cytotoxic chemotherapy for PBSC mobilization has achieved *in vivo* purging in most patients whose residual lymphoma cells were undetectable in the harvest products by polymerase chain reaction assay.[11,20] However, few studies have examined the effect of rituximab on mobilization and engraftment of auto-PBSC.[12,13] In general, factors associated with poor mobilization include extensive treatment before mobilization,[21] and rituximab administration may impair the efficiency of PBSC mobilization compared to that in the non-rituximab group. Hoerr *et al.*[13] reported that patients in the rituximab group had delays in platelet recovery post-transplant, but rituximab did not affect PBSC mobilization, and post-transplant neutrophil recovery, early complications, and mortality rates. In contrast, Benekli *et al.*[12] have shown the detrimental effect of rituximab on the mobilization and engraftment of PBSC because there was a significantly lower number in collected PBSC and a prolonged neutrophil recovery in the rituximab group. However, both studies were performed in patients with relapsed or refractory NHL who had been heavily treated previously, and the exact effect of rituximab on PBSC mobilization still remains unclear. Therefore, in the newly diagnosed DLBCL patients who were treated with the same treatment protocol consisting of chemotherapy with or without rituximab and auto-PBSCT as a primary treatment, we evaluated the characteristics of mobilization and transplantation of auto-PBSC. In our study, we found no disadvantage in the number of $CD34^+$ cells collected, recovery of neutrophils and platelets or post-transplant complications. As none of our patients were previously treated and this study was conducted as a primary therapy, mobilization potential might not have been impaired, resulting in no adverse effect of rituximab on the mobilization and engraftment of PBSC.

Mechanisms of PBSC mobilization may involve chemotherapy/G-CSF-induced modulation of chemokines, adhesion molecules and proteolytic enzymes.[14] Proteolytic enzymes such as neutrophil elastase, cathepsin G and matrix metalloproteinase-9 released from the activated neutrophils and monocytes can degrade and/or inactivate adhesion molecules such as VCAM-1/VLA-4, chemokines such as stromal-derived factor (SDF)-1/CXCR-4 and soluble Kit ligand, resulting in the disruption of contact between stem/progenitor cells and the bone marrow microenvironment, and then stem/progenitor cells would be released to migrate into peripheral blood.[14,15] However, recently late-onset neutropenia has been reported following rituximab-based chemotherapy,[22,23] and Dunleavy *et al.*[24] have suggested that rituximab may induce perturbations of SDF-1/CXCR-4 interaction, which could retard the egress of neutrophils from bone marrow. Therefore, we investigated expression levels of adhesion molecules on PBSC in the two groups. Low levels were documented in both groups, but we did not find any difference in expression levels of VLA-4, CXCR-4 and c-Kit on PBSC. Moreover, there was no difference in neutrophil counts, which might partially contribute to mobilization, at the time of PBSC collection, and the recovery in neutrophil and platelet counts was equally rapid following auto-PBSCT in the two groups. These results indicated that rituximab might not impair the mobilization as well as homing, engraftment and repopulation of PBSC, without altering the expression of adhesion molecules including at least VLA-4, CXCR-4 and c-Kit.

In summary, our data provide evidence that rituximab has no adverse effect on the mobilization and engraftment of PBSC, when rituximab is employed in the first-line treatment for newly diagnosed DLBCL patients. As rituximab received approval in September 2003 for the treatment of DLBCL in Japan, the median follow-up is too short to evaluate its effect on survival. Moreover, a high incidence of late-onset neutropenia following rituximab-containing chemotherapy and/or autologous stem cell transplantation has been reported, however, its mechanism still remains to be solved.[25,26] Larger studies and longer follow-ups are necessary to confirm these findings and to determine more optimal combinations of rituximab and auto-PBSCT as well as the impact of rituximab on disease-free survival in the treatment of NHL.