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De novo acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2): a clinicopathologic and cytogenetic study of an entity recently added to the WHO classification

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Acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) is a rare type of leukemia recently added to the World Health Organization (WHO) classification scheme. In this study, we analyzed the clinicopathologic and cytogenetic features of 30 cases of *de novo* acute myeloid leukemia with inv(3)/t(3;3). The median patient age was 53 years (range, 27–77 years). The platelet count was variable (range, 21–597 $\times 10^9$/l, median: 128 $\times 10^9$/l), and two (6.7%) patients presented with thrombocytosis ($>450 \times 10^9$/l). Morphologically, these neoplasms showed a spectrum of findings. Myelomonocytic differentiation was most common in 11 (37%) cases. Morphological evidence of dysplasia was observed in at least one lineage in 23 of 25 (92%) cases in which maturing elements could be assessed. In all, 5 (17%) patients had isolated inv(3) or t(3;3) and 25 (83%) patients had additional cytogenetic abnormalities, most often monosomy 7 (40%). Eleven (37%) patients had a complex karyotype ($\geq$3 additional abnormalities). *FLT3* gene mutation by internal tandem duplication was identified in 2 of 23 (9%) cases assessed. No clinical, pathological, or cytogenetic features independent of inv(3) or t(3;3) correlated with a worse outcome. However, patients treated with allogeneic stem cell transplantation ($n=11$) had a significantly better survival than did those treated with chemotherapy alone ($n=17$) (13.8 *vs* 8.0 months, $P=0.041$). We conclude that *de novo* acute myeloid leukemia associated with inv(3)/t(3;3) is an aggressive type of leukemia regardless of morphological or karyotypic findings, supporting the inclusion of this disease as a specific entity defined by inv(3)/t(3;3) in the WHO classification. Allogeneic stem cell transplantation seems to improve outcome in patients with this disease.
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Acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) was recently included in the World Health Organization (WHO) classification as a distinct type of leukemia under the umbrella term 'acute myeloid leukemia with recurrent cytogenetic abnormalities'.[1] Inv(3(q21q26.2) or t(3;3)(q21;q26.2) results in an inversion or homologous reciprocal translocation that leads to the juxtaposition of the ecotropic viral integration site-1 (*EVI1*) gene with the ribophorin 1(*RPN1*) gene. This rearrangement results in transcriptional activation of the *EVI1* gene,[2] which is believed to have a critical role in the pathogenesis of myeloid neoplasms, by driving cellular proliferation and/or impairing differentiation.[2,3]

Cases of acute myeloid leukemia with inv(3)/t(3;3) are rare and account for 1–2.5% of all acute myeloid leukemias.[4,5] Affected patients can present with *de novo* disease or the disease may arise as a result of progression from an underlying myelodysplastic syndrome. Relatively few reports of this disease are available in the literature, most of which emphasize the clinical aspects of this disease, including poor response to chemotherapy and poor prognosis of these patients.[4] In one study, bone marrow transplantation was reported to have a low curative potential.[6]

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In most studies to date, both patients with *de novo* disease and patients with preceding myelodysplastic syndrome have been grouped together.[1,7,8] The pathological features of acute leukemia with inv(3)/t(3;3) have also been underemphasized. For these reasons, we describe the clinicopathologic and cytogenetic features of 30 cases of *de novo* acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) treated at our institution and correlate the findings with outcome.

Materials and methods

Study Group

From 1 March 1995 to 31 May 2006, 30 *de novo* acute myeloid leukemia cases with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) were identified from the files of our hospital. The clinicopathologic features of this group were reviewed and compared with a group of 30 age-matched patients who had acute myeloid leukemia with a diploid karyotype. The median follow-up time for patients in this study was 288 days (range, 6–2028 days).

Morphological Analysis

Wright–Giemsa-stained bone marrow aspirate smears and hematoxylin–eosin-stained trephine biopsy and/or aspirate clot specimen slides were reviewed. Cytochemical stains for myeloperoxidase, butyrate esterase, and iron were performed using standard protocols. Each case was classified as acute myeloid leukemia according the criteria of the WHO 2008 classification.

Flow Cytometry Immunophenotyping

Four-color flow cytometry immunophenotypic analysis was performed on bone marrow aspirate samples using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). Incubation of cells with monoclonal antibodies at 4°C was followed by red blood cell lysis with NH_4Cl for 10 min and washing with phosphate-buffered saline solution. Cells were then resuspended and fixed with 1% formaldehyde. The panel of monoclonal antibodies included CD45 (conjugated with peridinin-chlorophyll-α-protein), CD2 (fluorescein isothiocyanate (FITC)), CD3 (allophycocyanin (APC)), CD7 (FITC or phycoerythrin (PE)), CD9 FITC, CD10 (FITC) (Immunotech, Fullerton, CA, USA), CD13 (PE), CD14 (APC), CD19 (FITC or APC), CD20 (APC), CD33 (PE), CD34 (FITC), CD38 (APC), CD52 (FITC) (Invitrogen, Carlsbad, CA, USA), CD64 (PE) (Caltag, Burlingame, CA, USA), CD117 (PE), and HLA-DR (FITC). All antibodies except those specified above were purchased from BD Biosciences. For each antibody, appropriate negative levels were determined by comparison with an isotype-matched control.

Conventional Cytogenetics

Metaphase cells from bone marrow aspirate samples were cultured for 24 and 48 h using previously described methods.[9] A methanol/glacial acetic acid fixation method was used for obtaining metaphase cells. Cell suspensions were dropped on air-dried slides, subsequently prepared, placed overnight in a 60°C oven, and G-banded using conventional methods. Twenty metaphases were analyzed.

FLT3 Mutation Analysis

In 23 cases, bone marrow aspirate material was available for *FLT3* mutation analysis. A fluorescence-based multiplex PCR assay was used to detect internal tandem duplication and D835 point mutations of the *FLT3* gene using DNA isolated from bone marrow aspirate or peripheral blood samples, as described previously.[10]

Statistical Analysis

Survival curves were calculated for overall survival using Kaplan–Meier analysis. Double-sided log-rank test was used to compare the overall survival between subgroups of patients. A fitted multivariate Cox proportional hazards analysis was used to assess the predictive effect of patient characteristics, including age, hemoglobin level, white blood cell count, platelet count, and blast percentage, on overall survival.

Results

Patient Characteristics

All patients were adults with a median age of 53 years (range, 27–77 years); 10 patients were under 40 years of age. There were 17 women and 13 men. Three patients presented initially to other institutions and underwent extensive workup and received some therapy before referral to our hospital.

The median hemoglobin level was 9.1 g/dl (range, 5.2–14.4 g/dl) (reference range, 14–18 g/dl for men and 12–16 g/dl for women). The median platelet count was 128×10^9/l (range, 21–597 $\times 10^9$/l) (reference range, 140–440 $\times 10^9$/l), and the median leukocyte count was slightly elevated at 12.8 (range, 1.2–178.8 $\times 10^9$/l reference range, 4–11 $\times 10^9$/l). Compared with an age-matched group of acute myeloid leukemia patients with a diploid karyotype, patients with inv(3)/t(3;3) presented with a higher hemoglobin level (median: 9.1 *vs* 7.7 g/dl, $P = 0.003$) and platelet count (128×10^9/l *vs* 48×10^9/l, $P = 0.038$). The leukocyte count was not significantly different between the two groups, $P = 0.44$) (Table 1).

Morphological and Flow Cytometric Findings

Morphologically, cases of acute myeloid leukemia with inv(3)/t(3;3) showed a wide spectrum of

findings. Using the French–American–British (FAB) system, M4 was the most common type (11 cases, 37%), followed by M0 (6 cases, 20%), M2 (6 cases, 20%), M1 (3 cases, 10%), M5 (1 case, 3%), and M7 (1 case, 3%). Two cases could not be FAB classified because of insufficient data. In 25 cases in which maturing elements were present and the presence of dysplasia could be assessed, dysplasia was observed in 23 cases (92%). Erythroid and megakaryocytic dysplasia were present in 16 cases each, whereas myeloid dysplasia was found 11 cases. Megakaryocytes were increased in number in nine cases, and showed dysplastic changes including small size and single to hypolobated nuclei (Figure 1).

Bone marrow cellularity was highly variable but mostly hypercellular with a median cellularity of 80% (range, 5–100%). In all, 20 of 28 (72%) cases

Table 1 Comparison of patient characteristics: AML-inv(3)/t(3;3) *vs* AML with a diploid karyotype

Median	*AML-inv(3)/t(3;3)*	*AML-diploid*	P-*value*
Patients	$n = 30$ [inv(3):21; t(3;3):9]	$n = 30$	
Age (years)	53 (27–77)	53 (27–77)	—
Gender	13 M/17 F	18 M/12 F	NS
WBC ($\times 10^9$/l)	12.8 (1.2–178.8)	7.6 (1.1–127)	0.44
HGB (g/dl)	9.1 (5.2–14.4 g/dl)	7.7 (4.2–11.6)	0.003
PLT ($\times 10^9$/l)	128 (21–597)	48 (7–708)	0.038

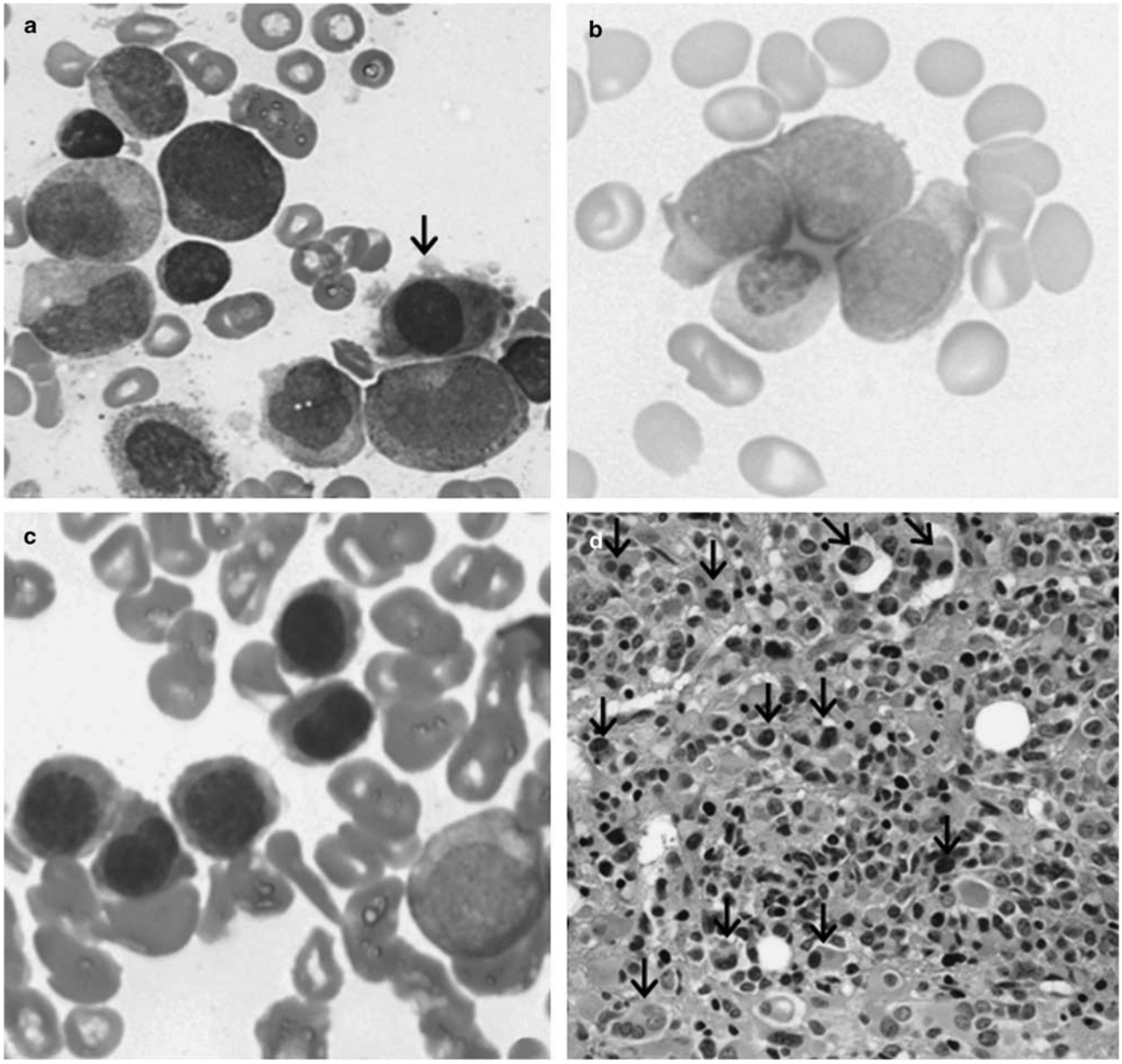

Figure 1 Morphological features of acute myeloid leukemia-inv(3)/t(3;3). (**a**) Acute myelomonocytic leukemia with a micromegakaryocyte (arrow) and dysplastic granulocytes; (**b**) acute myeloid leukemia without maturation showing a dysplastic granulocyte; (**c**) erythroid dysplasia; (panels **a**–**c**: bone marrow aspirate smears, Wright–Giemsa stain, $\times 1000$). (**d**) Trephine biopsy section, numerous dysplastic, and micromegakaryocytes (arrows) are present in a background of increased immature cells (hematoxylin and eosin, $\times 500$).

Table 2 Summary of characteristics of patients with AML-inv(3)/t(3;3)

BM dysplasia present ($n=25$)	23 (92%)
Bone marrow blasts (%)	69 (range 30–86)
Inv(3)/t(3;3) as sole abnormality	5 (17%)
Monosomy 7	12 (40%)
Complex cytogenetics	11 (37%)
FLT3-ITD mutation ($n=23$)	2 (9%)
Overall survival (years)	
1	33% (CI: 24–42%)
3	10% (CI: 5–15%)
5	3% (CI: 1–5%)

analyzed for myeloperoxidase by cytochemistry were positive, with 6–98% of blasts being positive. Iron stain was performed in two cases, and showed 28% ring sideroblasts in one case.

Flow cytometry immunophenotyping was performed in 27 cases and each case showed surface expression of CD13 and CD33 supporting myeloid lineage. Other markers expressed included HLA-DR ($n=26$; 96%), CD34 ($n=25$; 93%), CD117 ($n=24$; 89%), and CD38 (19/22; 86%). Six (22%) cases showed aberrant expression of CD7 and rare cases displayed aberrant surface expression of CD2, CD5, and CD9 (one case each). A subset of cases was positive for CD52 (11/18; 61%) or CD64 (5/28; 18%).

Cytogenetic and *FLT3* Results

Inv(3)(q21q26.2) was identified in 21 cases and t(3;3)(q21;q26.2) was identified in 9 cases. Inv(3)/t(3;3) was the sole aberration in five cases. One or more other chromosomal changes were identified in the remainder of the cases. The most frequent additional abnormality was monosomy 7 in 12 (40%) cases. Notably, monosomy 7 was part of a subclone in one case, indicating that monosomy 7 may have been a secondary event. A complex karyotype, defined as ≥ 3 cytogenetic abnormalities, was present in 11 (37%) cases in this cohort (Table 2). The *FLT*3 mutation by internal tandem duplication was identified in 2 of 23 (9%) cases analyzed.

Outcomes

Clinical follow-up was available for 28 patients, all of whom died within 67 months, with a median survival of 8.9 months (range, 0.2–67 months). The overall survival rate was 33% at 1 year, 10% at 3 years, and 3% at 5 years.

To assess the influence of patient characteristics on overall survival, a Cox regression analysis was performed with continuous variables, including age, hemoglobin levels, leukocyte count, platelet count, and bone marrow blast percentage. None of these features significantly correlated with overall survival in this study cohort.

Table 3 Fitted multivariate Cox proportional hazards model for overall survival ($n=30$)

Variable: yes vs *no*	P-*value*	*Hazard ratio*
Isolated inv(3)/t(3;3)	0.558	1.447
Monosomy 7	0.229	1.772
Complex cytogenetics	0.468	1.410
Allogeneic SCT	0.071	0.434

To determine whether the presence of additional cytogenetic abnormalities might influence patient outcome, a fitted multivariate Cox proportional hazards analysis was used to analyze overall survival in the presence or absence of monosomy 7 or a complex karyotype. There was no evidence that these abnormalities were associated with a worse outcome (monosomy 7: $P=0.229$; complex karyotype: $P=0.468$). When the five patients with isolated inv(3)/t(3;3) were compared with patients with inv(3)/t(3;3) plus additional abnormalities, no difference in overall survival was observed between the two groups ($P=0.558$). Additional cytogenetic abnormalities did not seem to contribute to a significantly worse survival (Table 3) (Figure 2).

We analyzed overall survival in this patient cohort subdivided according to therapy (chemotherapy alone *vs* allogeneic stem cell transplantation). There was a significant benefit of allogeneic stem cell transplantation, with a median overall survival of 13.8 months, in contrast to 8.0 months for patients treated with chemotherapy alone ($P=0.041$).

Discussion

Acute myeloid leukemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) has been recently added to the 2008 WHO classification in the chapter designated 'Acute myeloid leukemia with recurrent genetic abnormalities'. It is acknowledged in the WHO classification that acute myeloid leukemia cases with inv(3) or t(3;3) can either arise *de novo* or evolve from myelodysplastic syndrome. To date, relatively few studies on acute myeloid leukemia with inv(3)/t(3;3) are available in the literature. These studies have appeared in the clinical literature and have focused primarily on treatment and prognosis. These studies also have grouped together *de novo* tumors and cases that arose in patients who initially had myelodysplastic syndrome. The pathological findings of acute leukemia associated with inv(3)/t(3;3) have been underemphasized in the literature. In this study, we describe the clinicopathologic and cytogenetic features of 30 cases of *de novo* acute myeloid leukemia with inv(3) or t(3;3).

Morphologically, most cases demonstrated dysplastic changes in at least one cell lineage. The most distinctive change was in the megakaryocytic lineage. The megakaryocytes are generally small in size (micromegakaryocytes) with hypolobated nuclei

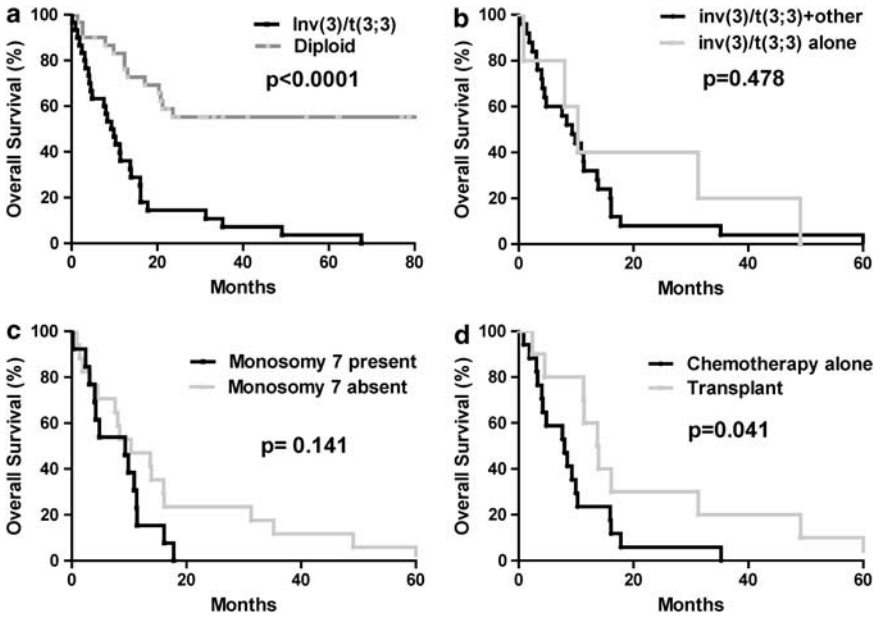

Figure 2 Survival curves comparing patients with inv(3)/t(3;3) with (**a**) diploid karyotype, (**b**) isolated inv(3)/t(3;3) *vs* others, (**c**) presence or absence of monosomy 7, and (**d**) impact of transplantation *vs* chemotherapy alone.

and mature-appearing cytoplasm actively producing platelets. This feature may be attributed to the activation of the *EVI1* gene, of which the proto-oncoprotein has been shown to block endomitosis in megakaryocytes and contribute to a defective megakaryocytic differentiation.[11] In addition, myelomonocytic differentiation is common (37% of cases), although most FAB subtypes can be observed from M0 to M7.

The *EVI1* gene, and its longer splicing variant *EVI1-MDS1*, are products of *MECOM* (MDS1 and EVI1 complex) located on chromosome 3q26.2 that encodes a highly conserved proto-oncoprotein in humans, which may function as a transcription-regulatory protein.[12] *EVI1* encodes a 1051 amino-acid zinc-finger protein of 145 kDa. It has been shown that EVI1 affects multiple signaling pathways, including the TGF-β, c-Jun N-terminal kinase-1 (JNK1), and activator protein-1 (AP1) pathways.[13] The expression of EVI1 is important in organogenesis, and deregulation has been implicated in the pathogenesis of leukemia and other hematopoietic neoplasms.[14] Inversion or translocation of chromosome 3q21q26.2 results in transcriptional activation of the *EVI1* gene and in most cases, overexpression of EVI1.[2] Recently, elevated levels of *EVI1* have been shown to predict adverse outcome and poor response to therapy in acute myeloid leukemia patients.[15,16]

Unlike some other types of acute myeloid leukemia with recurrent genetic abnormalities, additional cytogenetic abnormalities are common in acute myeloid leukemia associated with inv(3)/t(3;3). Only five cases in this study cohort had inv(3)/t(3;3) as a sole cytogenetic abnormality. The most common additional abnormality in this series was monosomy 7, detected in 40% of cases, followed by a complex karyotype in approximately one-third of the cases. In this series, 5q lesions were also detected in five cases, another abnormality commonly reported in acute myeloid leukemia associated with inv(3)/t(3;3). It has been postulated that monosomy 7 or 5q lesions precede inv(3)/t(3;3). However, one of our cases showed monosomy 7 present as a subclone, suggesting that inv(3)/t(3;3) is an initial event. This is supported by the observation that insertional activation of *EVI1* in human cells induces genomic instability and monosomy 7 reported by Stein *et al*.[17] None of these additional abnormalities seemed to affect overall survival, suggesting that the presence of inv(3)/t(3;3) alone predicts a poor prognosis. It is possible that additional molecular genetic events that are not readily detectable by conventional cytogenetic analysis are also implicated in cases of apparently isolated inv(3)/t(3;3). The low incidence of the *FLT3* mutation by internal tandem duplication ($\sim$9%) in this cohort further indicates that the poor outcome of affected patients is unlikely due to the *FLT3* mutation in this type of acute leukemia.

Patients with acute myeloid leukemia associated with inv(3)/t(3;3) have a dismal prognosis. In our study, overall survival at 1 year was barely 33%, which further decreased to 3% at 5 years. Patients are often refractory to chemotherapy and only 12 of 28 (42%) patients with follow-up in this study achieved complete remission, sometimes often after multiple rounds of treatment. All patients died within 67 months of diagnosis. This poor outcome was not affected by age, peripheral blood count at diagnosis, bone marrow blast count, or the presence of additional cytogenetic abnormalities. Patients treated with allogeneic stem cell transplantation seemed to have a better response in this patient cohort. The median survival was 13.8 months for patients treated with

stem cell transplantation, 5.8 months longer than the survival of patients treated with chemotherapy alone ($P = 0.041$). The survival benefit observed in patients treated with stem cell transplantation is similar to that reported by Weisser *et al*.[4] However, the overall survival was shorter in our study (median: 13.8 months in our study *vs* 899 days) (or ~30 months by Weisser *et al*). This difference may be explained, in part, by the fact that our hospital is a referral center where patients with refractory diseases are more often encountered.

Acute myeloid leukemia with inv(3)/t(3;3) is primarily a disease of adults. Patient age ranged from 27 to 77 years. The median age of 53 years was younger than that of our control group of patients who had a diploid karyotype, 63 years. Compared with the control group, patients with inv(3)/t(3;3) presented with a higher hemoglobin level, and higher leukocyte and platelet counts. The latter has been noted as a characteristic of acute myeloid leukemia associated with inv(3)/t(3;3).[1,4,7] This is at least partially due to abnormal megakaryopoiesis associated with inv(3)/t(3;3).[11,18] However, none of these factors affected the overall survival in a multivariable analysis.

In summary, our data emphasized that *de novo* acute myeloid leukemia associated with inv(3)/t(3;3) is an aggressive form of leukemia with a poor response to conventional chemotherapy and poor prognosis. No clinical, pathological, or cytogenetic findings independent of inv(3)/t(3;3) correlated with differences in overall survival. Therefore, our data support the importance of inv(3)/t(3;3) abnormality and the decision to include this disease in the 2008 WHO classification as a type of acute myeloid leukemia defined, in large part, by the presence of this cytogenetic abnormality. The presence of micromegakaryocytes in over half of these cases is a useful clue to considering the diagnosis. Our data are preliminary, but they suggest that allogeneic stem cell transplantation may be beneficial for patients with this disease.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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