

Acute myeloid leukemia with t(3;21)(q26.2;q22) developing following low-dose methotrexate therapy for rheumatoid arthritis and expressing two AML1/MDS1/EVI1 fusion proteins: A case report

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Abstract. The t(3;21)(q26.2;q22) translocation is a rare chromosomal abnormality exhibited almost exclusively in therapy-related myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML) or in the blastic crisis phase of chronic myelogenous leukemia, which results in the fusion of the runt related transcription factor 1 (*RUNX1*, also called *AML1*) gene at 21q22 to the myelodysplasia syndrome 1 (*MDS1*)-ecotropic virus integration site 1 (*EVI1*) complex locus (*MECOM*) at 3q26.2, generating various fusion transcripts, including *AML1/MDS1/EVI1* (*AME*). The present study examined the case of an 84-year-old Japanese woman who developed t-MDS/AML with t(3;21)(q26.2;q22) subsequent to receiving low-dose methotrexate (MTX) treatment for rheumatoid arthritis. Following treatment with MTX for 6 years, the patient developed anemia and neutropenia, and MTX was discontinued. A total of 3 years later, the patient was diagnosed with MDS with t(3;21)(q26.2;q22) and del(5q), which progressed rapidly to AML within 3 months. The patient was subsequently treated with azacitidine and cytarabine chemotherapy, but succumbed to the disease 6 months after diagnosis. Sequencing analysis of the nested reverse transcription-PCR products from the leukemic cells revealed the expression of two types of alternatively-spliced *AME* transcripts with or

without *RUNX1* exon 6 sequences. Western blot analysis of the leukemic cells of the patient additionally revealed that the corresponding *AME* fusion protein products were expressed at high levels, and that these cells also prominently expressed CCAAT/enhancer-binding protein α , the repression of which has been reported to be involved in leukemogenesis mediated by *AME*. To the best of our knowledge, the case discussed in the present study represents the first report of MDS/AML with t(3;21)(q26.2;q22) developing following low-dose MTX therapy for rheumatoid arthritis. Nonetheless, the clinical and molecular features of the patient in the present study were representative of those patients who typically develop this disease following exposure to chemotherapy or radiotherapy for primary malignancy, which implicates MTX in the pathogenesis of t-MDS/AML. Moreover, we confirmed the expression of two *AME* fusion proteins for the first time in primary leukemic cells and analyzed several cellular factors implicated in *AME*-mediated leukemogenesis to gain some insight into its molecular mechanisms.

Introduction

A total of ~10% of acute myeloid leukemia (AML) develops following exposure to chemotherapy and/or radiation for a different primary malignancy and is categorized as therapy-related AML (t-AML) in the current World Health Organization (WHO) classification (1). The most common type of t-AML, which occurs subsequent to exposure to alkylating agents and/or radiation with a latency period of 5-10 years, is frequently preceded by therapy-related myelodysplastic syndrome (t-MDS) and is accompanied with an unbalanced loss of genetic material, often involving chromosome 5 and/or 7 (1). The less common type of t-AML, occurring following treatment with agents targeting topoisomerase II, exhibits a shorter latency period of 1-5 years without the preceding myelodysplastic phase and is often associated with balanced recurrent chromosomal translocations, frequently involving the mixed-lineage leukemia 1 (*MLL*) gene located at 11q23 or the runt related transcription factor 1 (*RUNX1*, or

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alternatively designated as *AML1*) gene at 21q22 (1). Although antimetabolites, including fludarabine, are also implicated in therapy-related hematological malignancies, it remains unknown if methotrexate (MTX), particularly when used at a low dose for the treatment of rheumatoid arthritis, serves a role in development of MDS/AML.

The t(3;21)(q26.2;q22) translocation is a rare chromosomal abnormality exhibited in <1% of AML/MDS, primarily t-MDS/AML or in the blastic crisis phase of chronic myelogenous leukemia (2-5). Chromosome 3q26.2 abnormalities typically activate the ecotropic virus integration site 1 (*EVII*) gene, which has been implicated in the pathogenesis of AML and is associated with a poor prognosis (6-8). *EVII* is a nuclear transcription factor implicated in the regulation of proliferation and maintenance of hematopoietic stem cells. *EVII* also exists as the fusion protein *MDS1/EVII* (ME), which is generated by the alternative splicing of the third exon of *MDS1* to the second exon of *EVII* with the two genes located nearby to form the *MDS1-EVII* complex locus (*MECOM*) on 3q26.2 (6,7). The most common types of 3q26.2 abnormalities are inv (3)(q21q26.2)/t(3;3)(q21;q26.2), which define a category of AML in the WHO classification and relocate a distal enhancer of the GATA binding protein 2 (*GATA2*) gene to a region between *MDS1* and *EVII*, which ectopically activates *EVII* (1,7,9,10). In contrast, t(3;21)(q26.2;q22) results in the translocation of a part of *RUNX1* at 21q22 to *MECOM* and generates various fusion transcripts (2,4). Although these fusion transcripts, including *AML1/MDS1/EVII* (*AME*), have been analyzed in several cases of myeloid neoplasms associated with t(3;21)(q26.2;q22), the expression of the aberrant fusion protein products in primary leukemic cells has not previously been confirmed (3,11-16).

The present study examined a patient with MDS/AML with t(3;21)(q26.2;q22) that developed not with exposure to chemotherapy or radiotherapy for malignancy, but following treatment with low-dose MTX for rheumatoid arthritis. The fusion transcripts of *RUNX1/MECOM* were analyzed, and two alternatively-spliced *AME* transcripts with or without *RUNX1* exon 6 sequences were identified. Additionally, the expression of the protein products of two of these fusion transcripts was confirmed, and the expression of CCAAT/enhancer-binding protein α (CEBP α) and other proteins implicated in *AME*-mediated leukemogenesis in leukemic cells from the patient was evaluated.

Case report

Materials and methods

Ethics statement. The present study was approved by the Ethical Committee of Tokyo Medical and Dental University (Tokyo, Japan). Written informed consent was obtained from the patient in compliance with the Declaration of Helsinki.

Nested reverse transcription-polymerase chain reaction (RT-PCR) and sequencing analyses of the *AME* fusion transcripts. The peripheral blood mononuclear cells from the patient were obtained by Ficoll-Hypaque density gradient centrifugation (at 500 x g for 15 min at room temperature). RNA samples were prepared from the peripheral blood mononuclear cells of the patient or HL60 cells, used as a

negative control, using the RNeasy[®] Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol, and subjected to RT-PCR using *RUNX1* and *EVII* primers or primers for β -*actin* as a control. The RT-PCR for *AME* transcripts were performed as described previously (11) using the following primers: *RUNX1* external forward, 5'-AAGTCG CCACCTACCACAGA-3' and internal forward, 5'-GCCATC AAAATCACAGTGGGA-3'; and *EVII* external reverse, 5'-CCG GCGCCATAGTTTCATGC-3'; and internal reverse, 5'-GGA TAGTCTTCGCTCTTCAT-3'. The primers used for β -*actin* were: Forward, GGAGAAGCTGTGCTACGTCGCC; and reverse, TACATGGTGGTGCCGCCAGACAG. The RT-PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide. For direct sequencing analysis of the *AME* fusion transcripts, two species of nested RT-PCR products were isolated from the gel using the MinElute[®] Gel Extraction kit (Qiagen GmbH), according to the manufacturer's protocol, and sequenced using the same internal primers.

Western blot analysis. The peripheral blood mononuclear cells from the patient and human leukemic HEL and MOLM-1 cells, obtained from the Fujisaki Cell Center (Okayama, Japan) and cultured in RPMI-1640 medium (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) containing 10% fetal calf serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C in a 5% CO₂ incubator, were lysed and subjected to western blot analysis as described previously (17). Antibodies directed against *RUNX1* (catalog no., CS4336), *EVII* (catalog no., CS2593) and calreticulin (catalog no., CS12238) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies directed against CEBP α (catalog no., SC61) and heat shock protein 90 (HSP90; catalog no., SC13119) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). These first antibodies were used at 0.2 μ g/ml at 4°C in overnight incubation, while the relevant secondary antibodies (HRP-linked anti-rabbit or -mouse IgG; NA934 or NA9310, respectively, from GE Healthcare Japan, Tokyo, Japan) were used at 0.2 μ g/ml at room temperature for 1 h.

Case results. An 84-year-old Japanese woman was referred to the Department of Hematology of the Graduate School of Medical and Dental Sciences at Tokyo Medical and Dental University (Tokyo, Japan) in September 2013 due to anemia and the appearance of blasts in the peripheral blood. The patient had been diagnosed with rheumatoid arthritis in 1997 and subsequently treated with bucillamine from 1997 until unidentified time, actarit between August 2001 and February 2002, salazosulfapyridine between February 2002 and September 2002 and penicillamine between September 2002 and June 2005, although clinical information on the dosages of these drugs was not available. In 2004, treatment with low-dose MTX (weekly doses of 6-10 mg, adjusted by clinical and laboratory findings, divided into 2 or 3 doses orally every 12 h) and prednisolone was started. In 2006, the patient was diagnosed with lung cancer and underwent radical surgery without any chemotherapy or radiotherapy. As the patient developed anemia and neutropenia in August 2010, MTX was discontinued and substituted for tacrolimus (daily doses of 1-2 mg, adjusted by clinical and laboratory findings, orally once a day). The duration of the MTX therapy was 75 months

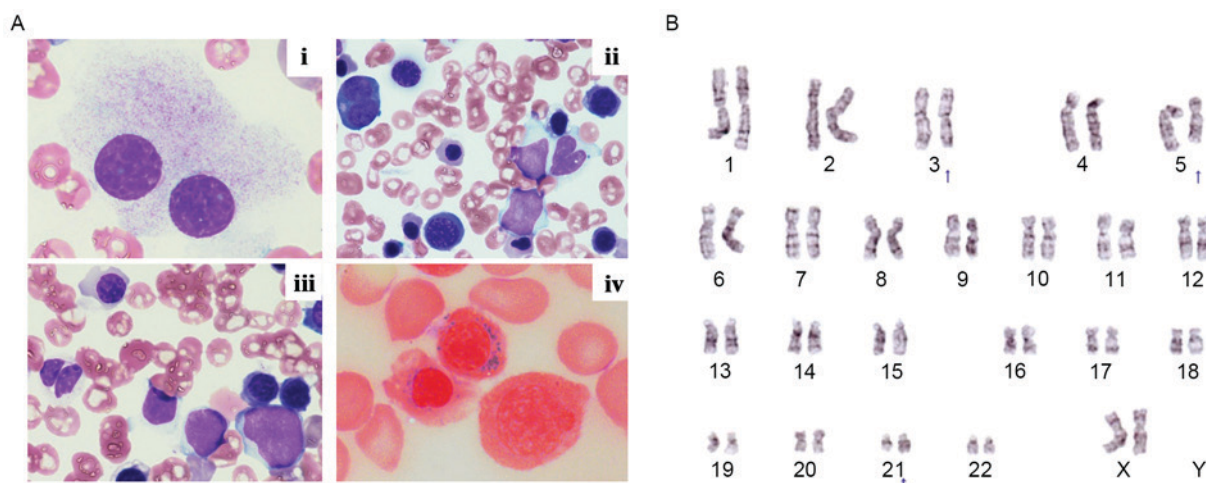


Figure 1. Morphological and cytogenetic analyses of bone marrow cells at diagnosis of myelodysplastic syndrome. (A) Bone marrow smear illustrating i) a megakaryocyte with separated nuclei; ii and iii) dysplastic erythroblasts, increased blasts, pseudo-Pelger-Huet anomaly and degranulation of neutrophils and iv) ringed sideroblasts. (B) A Giemsa-banded karyogram demonstrating 46,XX,t(3;21)(q26.2;q22),del(5)(q?). Abnormal chromosomes are indicated by arrows.

and the accumulated dose was 2,204 mg. Despite stopping MTX treatment, the patient's anemia gradually progressed, with the hemoglobin level decreasing to 6.6 g/dl (normal range, 12.0-15.0 g/dl) in September 2013, when myeloblasts and hypogranular neutrophils appeared in the peripheral blood.

On the patient's first visit to the Department of Hematology (Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University) the white blood cell count was $3.7 \times 10^9/l$ (normal range, $3.6-9.3 \times 10^9/l$) with an appearance of 3% blasts, the hemoglobin level was 6.1 g/dl, and the platelet count was $155 \times 10^9/l$ (normal range, $120-410 \times 10^9/l$). Bone marrow examination demonstrated slightly hypercellular marrow with 6.2% blast (normal range, 0.1-0.7%), pseudo-Pelger-Huet anomaly, hypogranular neutrophils, megaloblastoid changes, increased ringed sideroblasts (31%), and micromegakaryocytes (Fig. 1A). Flow cytometric analysis demonstrated that the blasts were positive for cluster of differentiation (CD)13 (82.2%), CD33 (43.2%, dimly expressed), and CD34 (41.7%), and partially positive for CD7 (18.5%). Cytogenetic analysis illustrated that the karyotype of the bone marrow cells was as follows: 46,XX,t(3;21)(q26.2;q22)[1]/46,idem,del(5)(q?) (16) (Fig. 1B). The position of deletion in 5q [(5)(q?)] could not be determined more precisely most likely because of the quality of metaphase samples obtained. Thus, t(3;21)(q26.2;q22) was observed in all the cells in metaphase (17) that were analyzed. In addition, the RT-PCR analysis using *AML1* and *EVII* primers revealed two AME bands in gel electrophoresis (Fig. 2A). These RT-PCR products were isolated from the agarose gel and sequenced, which revealed two types of *AML1/MDS1/EVII* fusion transcripts, in which either exon 5 or 6 of *RUNX1* was directly fused with exon 2 of *MECOM*, as demonstrated in Fig. 2B. Consistent with this, two AME fusion proteins with apparent molecular masses of 210 and 186 kDa were detected in bone marrow mononuclear cells by western blotting with antibodies directed against *EVII* and *RUNX1* (Fig. 2C). *EVII* was detectable in HEL and MOLM-1 cells, but not in the patient's leukemic cells, which also expressed *RUNX1*, *CEBP α* and calreticulin at readily detectable levels.

The patient was diagnosed with MDS: Refractory anemia with excess blasts [international prognostic scoring system (IPSS) (18): Int-1; revised IPSS (19): High; WHO adapted prognostic scoring system (20): High]. Although treatment with oral metenolone acetate (15 mg/day) was started in October 2013, the patient's anemia progressed and blasts in the peripheral blood increased. In December 2013, a bone marrow examination revealed 25.2% blasts, leading to the diagnosis of AML with myelodysplasia-related changes. In January 2014, treatment with azacitidine (75 mg/m²/day for 7 days) was started; however, the patient's cytopenia progressed (the white blood cell count, $2.2 \times 10^9/l$; platelet count, $43 \times 10^9/l$) and the bone marrow blasts increased. The patient was subsequently treated with low-dose cytarabine chemotherapy (10 mg/m²/day), but succumbed to the disease in July 2014.

Discussion

The present study described a case of AML with t(3;21)(q26.2;q22) that developed from MDS following treatment with low-dose MTX for rheumatoid arthritis. MDS/AML with t(3;21)(q26.2;q22) has been identified almost exclusively as t-MDS/AML. For instance, a large case series examined by the University of Texas MD Anderson Cancer Center (Houston, TX, USA) revealed that 16/17 cases (94.1%) of t(3;21)(q26.2;q22)-positive MDS/AML had previously received chemotherapy or radiotherapy (5). It is well established that other balanced recurrent chromosomal translocations in t-AML, including those involving *RUNX1*, are typically observed following treatment with topoisomerase II inhibitors without the antecedent MDS phase or myelodysplasia-related morphological changes, and are associated with a better prognosis compared with other types of t-AML (1). Notably, a previous study by the same group at the University of Texas MD Anderson Cancer Center implicated antimetabolites in the pathogenesis of t-MDS/AML with t(3;21)(q26.2;q22), as prior treatment with the pyrimidine analog 5-fluorouracil or the purine analog fludarabine was identified in 6/8 patients with this type of t-MDS/AML (3). However, the subsequent study revealed that a number of the

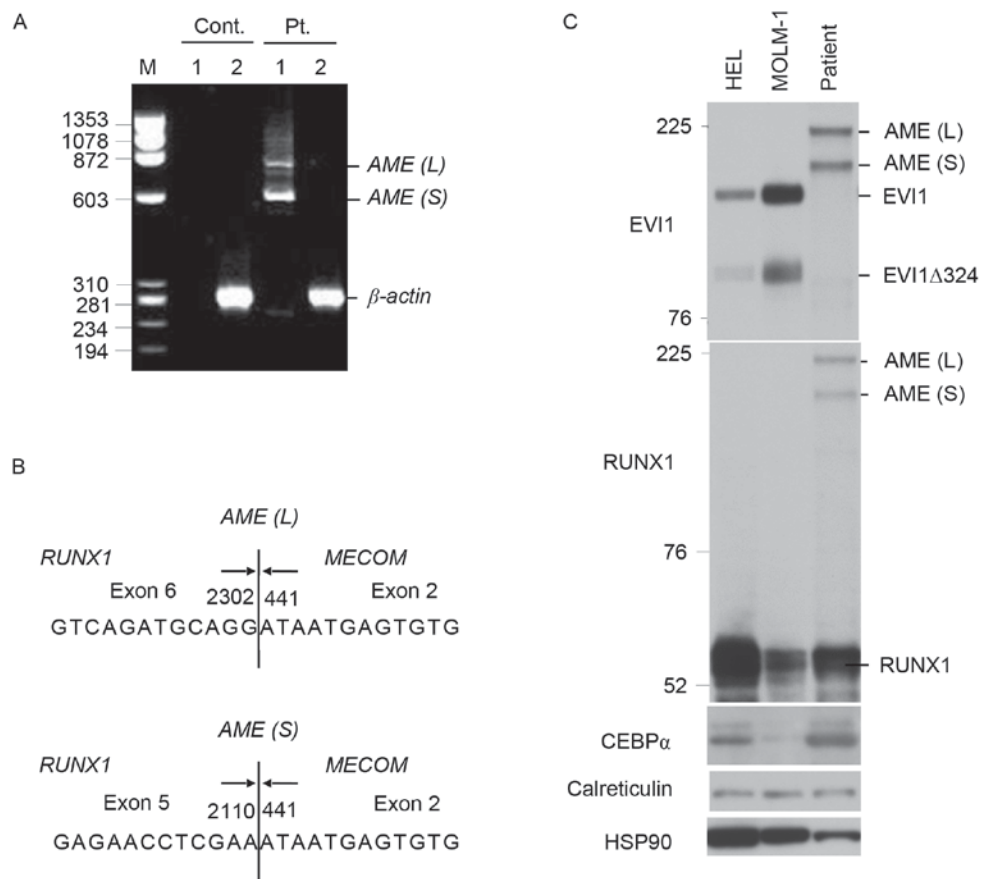


Figure 2. Analyses of *AME* fusion transcripts and proteins in the leukemic cells of the patient. (A) Reverse transcription (RT)-PCR analyses of the *AME* fusion transcripts. M, marker lane; 1, products from *RUNX1* and *EVI1* primers; 2, β-actin control. Sizes are given in bp. Pt., patient's bone marrow cells; Cont., HL60 cells; *AME*, *AML/MDS1/EVI1*. The positions of longer and shorter *AME* (*AML/MDS1/EVI1*) fusion transcripts, *AME (L)* and *AME (S)*, as well as that of β-actin are also indicated. (B) Sequence analysis of the *AME* fusion transcripts obtained from RT-PCR. Arrows illustrate the boundaries of exons, with the nucleotide number for *RUNX1* (NM_001001890.2) and *MECCOM* (NM_004991.3) indicated. Vertical lines indicate the junctions of the chimeric transcripts. (C) Western blot analysis of the *AME* fusion proteins in patient's leukemic cells. Total cell lysates from HEL cells, MOLM-1 cells, or patient's bone marrow cells, as indicated, were subjected to Western blot analysis using antibodies against indicated proteins. Sizes are given in kDa. Positions of larger and smaller *AME* fusion proteins, *AME (L)* and *AME (S)*, as well as those for *EVI1*, the smaller form of *EVI1* lacking 324 amino acids (*EVI1*Δ324), and *RUNX1* are also indicated. HSP90 was used for a protein loading control.

16 patients with t(3;21)(q26.2;q22)-positive t-MDS/AML also received alkylating agents and DNA topoisomerase II inhibitors for the treatment of their primary tumors (5). Thus, in contrast to t-AML associated with other balanced recurrent translocations, t-MDS/1-AML with t(3;21)(q26.2;q22) was observed subsequent to treatment with wide-ranging chemotherapeutics and demonstrated a very short overall survival rate (median, 4.7 months) (5). In the present study, the patient exhibited typical clinical and morphological features of MDS/AML with t(3;21)(q26.2;q22), and survived only 10 months following the diagnosis of MDS. However, to the best of our knowledge, t(3;21)(q26.2;q22)-positive MDS/AML following treatment with MTX has never been reported, and cannot be identified in the Mitelman Database of Chromosome Aberrations and Gene Fusion in Cancer (21).

It is well established that treatment with low-dose MTX for rheumatoid arthritis is associated with the development of lymphoproliferative disorders (LPDs), which are frequently associated with Epstein Barr virus infection and often regress spontaneously following the discontinuation of MTX (1,22). Thus, immunodeficiency caused by the MTX therapy is strongly implicated in the pathogenesis of LPDs. Conversely, it remains

unknown whether MTX therapy for rheumatoid arthritis may serve a role in pathogenesis of MDS/AML. To the best of our knowledge, the cases of 13 patients with AML developing following low-dose MTX treatment for rheumatoid arthritis have previously been reported (Table I) (23-29). However, the clinical features, including latency periods, cumulative dosages of MTX and the presence or absence of antecedent MDS and myelodysplasia-related morphological changes have been heterogeneous among these patients. Cytogenetic changes observed in these patients have also been diverse; while complex karyotypic abnormalities were exhibited in one patient, t(8;21) was observed in two other cases (24,25,29). Conversely, the patient in the present study exhibited typical clinical and morphological features of MDS/AML associated with t(3;21)(q26.2;q22), which develops almost exclusively as t-MDS/AML (3,5). This indicates that low-dose MTX therapy is causally associated with the development of MDS/AML in the case reported in the present study, although it remains unknown whether the mechanism underlying this association involves DNA mutagenesis induced by MTX as an antimetabolite or the immunosuppressive effect of MTX. In this regard, it should be noted that rheumatoid arthritis itself has

Table I. Identified cases of MDS/AML developing following low-dose MTX therapy for RA.

Age/sex	Duration of RA (y)	Total dose of MTX (mg)	Diagnosis	Cytogenetic abnormality	(Refs.)
83/F	33	690	AML	46,XX	(23)
60/F	23	80	AML-M2	t(8;21)	(24)
NA	0.6	NA	CMML to AML-M2	NA	(25)
NA	11	NA	MDS/AML-M2	t(8;21)	(25)
NA	10	NA	AML-M4	NA	(25)
71/M	16	750	AML-M1	46,XY	(26)
72/F	5	1200	AML-M4	NA	(26)
52/M	2	1250	AML-M0	47,XY,+13	(26)
70/F	9	500	AML-M5	46,XX	(26)
68/F	11	1700	AML-M6	46,XX	(27)
73/F	15	5850	AML with MRC	46,XX	(28)
35/F	3	1170	AML with MRC	46,XX	(28)
78/F	10	NA	Myeloid sarcoma	Complex	(29)
84/F	17	2204	MDS/AML	t(3;21),del(5)(q?)	*

RA, rheumatoid arthritis; MTX, methotrexate; NA, not available; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; AML-M0, undifferentiated AML; AML-M1, AML with minimal maturation; AML-M2, AML with maturation; AML-M4, acute myelomonocytic leukemia; AML-M5, acute monocytic leukemia; AML-M6, acute erythroid leukemia; MRC, myelodysplasia-related change; M, male; F, female; *, the present case.

been associated with an elevated risk of AML [odds ratio (OR) 1.28, 95% confidence interval (CI) 1.11-1.47] and MDS (OR 1.52, 95% CI 1.27-1.81) (30). Additionally, rheumatoid arthritis is frequently treated with immunosuppressants in addition to MTX, as is the case for the patient in the present study who had received tacrolimus after stopping MTX. This suggests that patients with rheumatoid arthritis who are treated with MTX need to be carefully monitored for the development of MDS/AML. Further reports of patients with t-MDS/AML associated with MTX are required to clarify the etiological and clinical significance between MTX and t-MDS/AML.

The t(3;21)(q26.2;q22) translocation primarily generates the fusion gene transcript *AME*, although transcripts of *RUNX1* fused directly with *EVII* or *RPL22* near *MECOM* have also been identified (2,11,12,31). In the patient discussed in the present study, two *AME* fusion transcripts corresponding with *RUNX1* fused at its 3' end of exon 5 or 6 to the 5' end of exon 2 of *MECOM* were detected. Thus, these fusion transcripts should encode the N-terminus of *RUNX1* containing the DNA-binding Runt domain fused to the majority of the *MDS1/EVII* protein. These two different transcripts, presumably generated by alternative splicing, represent the two predominant forms of *AME*, which have previously been identified in the t(3;21)-carrying leukemic cell line SKH1 and in primary leukemic cells (3,11-16), with a few patients revealed to express both forms (14,16). However, the expression of the protein products of these fusion transcripts in primary leukemic cells has not yet been documented, to the best of our knowledge. In the present study, it was confirmed that the fusion products were expressed in present patient at a high level, comparable to that of *EVII* in human leukemic MOML-1 cells with inv(3)(q21.2;q26) (7,9) and HEL cells (32).

The Runt domain maintained in *AME* is the DNA-binding domain of *RUNX1*, which is the DNA-binding subunit of the core-binding transcription factor that regulates the expression of genes essential for hematopoiesis. By recruiting histone deacetyltransferase and transcriptional co-repressors through the C-terminal domain of *EVII*, *AME* represses the *RUNX1*-dependent promoters in a dominant-negative manner, thus serving an essential role in leukemogenesis (2,4). This is consistent with the observation of the present study that the leukemic cells of the patient did not express *EVII* despite the fact that wild-type *RUNX1* was expressed at a readily detectable level, since *EVII* has been suggested to be a target gene of *RUNX1* (32).

AME has also been demonstrated to serve a role in leukemogenesis through the inhibition of *CEBPα*, a transcription factor that regulates the expression of genes associated with myeloid differentiation, including its own gene (2,4,31,33). Helbling *et al* (33) previously suggested that the conditional expression of *AME* in the myeloid leukemic cell line U937 suppressed the expression of *CEBPα* through the induction and activation of calreticulin, a putative inhibitor of *CEBPα* translocation. The authors additionally demonstrated that leukemic cells from none of the 8 patients with t(3;21)-positive AML examined expressed *CEBPα*, although the expression of *AME* in these cells was not confirmed (33). By contrast, *CEBPα* was readily detectable in the leukemic cells from the patient in the present study. In addition, the expression of calreticulin was not distinctively enhanced in the present study, although the possible activation of calreticulin in the leukemic cells of the patient was not examined. Conversely, Tokita *et al* (31) revealed that *AME* inhibited *CEBPα*-mediated transcriptional activity in the murine hematopoietic progenitor cell line LG-3,

although the mRNA levels of *CEBPA*, a target gene of *CEBPA* itself, and the expression level of calreticulin was not altered by the expression of AME in these cells. These observations suggest that the effects of AME on various cellular factors and events associated with leukemogenesis may depend on the cellular context. Thus, a detailed analysis of these factors, particularly at the protein level, in primary leukemic cells is warranted to generate more data in regards to the mechanism of AME-mediated leukemogenesis.

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