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Yamamoto, Katsuya ; Yakushijin, Kimikazu ; Ichikawa, Hiroya ; Okamura, Atsuo ; Nagao, Shigeki ; Kakiuchi, Seiji ; Kurata, Keiji ; Kawamoto,…

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LETTER TO THE EDITOR

Coexpression of *ETV6/MDS1/EVI1* and *ETV6/EVI1* fusion transcripts in acute myeloid leukemia with t(3;12)(q26.2;p13) and thrombocytosis

Katsuya Yamamoto[a], Kimikazu Yakushijin[a], Hiroya Ichikawa[a], Atsuo Okamura[a], Shigeki Nagao[a], Seiji Kakiuchi[a], Keiji Kurata[a], Shinichiro Kawamoto[a], Keiji Matsui[b], Yuji Nakamachi[b], Jun Saegusa[b], Hiroshi Matsuoka[b] and Hironobu Minami[b]

[a] Division of Medical Oncology/Hematology, Department of Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

[b] Department of Clinical Laboratory, Kobe University Hospital, Kobe, Japan

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Corresponding author: Katsuya Yamamoto

Address: Division of Medical Oncology/Hematology, Department of Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

TEL: +81-78-382-5820

FAX: +81-78-382-5821

E-mail: kyamamo@med.kobe-u.ac.jp

Chromosomal rearrangements involving the *MECOM* locus, which consists of two genes *MDS1* and *EVI1* located at 3q26.2, are found in different types of myeloid malignancies [1,2]. Among these, the t(3;12)(q26.2;p13) translocation is a rare but recurrent aberration in acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), and chronic myeloid leukemia (CML). It is usually associated with megakaryocytic dysplasia, multilineage involvement, and a very poor prognosis [3-5]. More than 40 cases with t(3;12) have been reported, but only six cases have been molecularly analyzed [6-11]. This translocation is shown to produce the *ETV6/MDS1/EVI1* and/or *ETV6*/*EVI1* fusion transcripts. The *ETV6* gene exon 2 at 12p13 is fused to *MDS1/EVI1*, which results from splicing of the *MDS1* exon 2 to the *EVI1* exon 2, whereas the latter results from the direct fusion between the *ETV6* exon 2 and *EVI1* exon 2. Coexpression of both transcripts has been shown in only one CML case [7]. The resultant fusion proteins have oncogenic potential since the *ETV6* promoter active in hematopoietic cells could induce inappropriate expression of the transcription factor EVI1 [3,7]. Here, we describe an unusual case of AML with t(3;12)(q26.2;p13) expressing both transcripts and thrombocytosis. The translocation constantly led to high *ETV6/MDS1/EVI1* and low *ETV6/EVI1* expression levels, suggesting the pathological significance of the former transcript.

A 21-year-old man was referred to our hospital because of thrombocytopenia. Peripheral blood showed hemoglobin 13.9 g/dl, platelets 86×10^9/l and leukocytes 4.1×10^9/l with 43% segmented neutrophils, 1% eosinophils, 10% monocytes, and 46% lymphocytes. The bone marrow was normocellular with increased numbers of megakaryocytes: 2.4% blasts, 35.0% mature myeloid cells, 6.0% monocytes, 30.6% lymphocytes, and 22.2% erythroblasts. Megakaryocytic dysplasia including micromegakaryocytes was found. We made a diagnosis of MDS with single lineage dysplasia. Peripheral blood counts 20 months after the initial diagnosis were hemoglobin 12.9 g/dl, platelets 84×10^9/l and leukocytes 2.7×10^9/l with no

blasts. Thereafter, anemia gradually progressed, and he received red blood cell transfusions repeatedly. He did not receive any other treatment including chemotherapy.

Five years after diagnosis, he was admitted to our hospital because of an increased number of blasts and thrombocytosis in the peripheral blood: hemoglobin 4.6 g/dl, platelets 605×10^9/l and leukocytes 3.9×10^9/l with 28% blasts, 1% metamyelocytes, 5% band forms, 41% segmented neutrophils, and 25% lymphocytes. The bone marrow was infiltrated with 41.0% blasts, and other differential counts were 0.2% promyelocytes, 1.0% myelocytes, 1.0% metamyelocytes, 5.0% band forms, 19.6% segmented neutrophils, 3.2% monocytes, 20.6% lymphocytes, and 4.6% erythroblasts. Trilineage dysplasia, including giant platelets, mononuclear micromegakaryocytes, and megaloblastic erythroblasts, was evident (Figure 1A). Immunophenotyping with flow cytometry using CD45/side scatter gating revealed that the gated leukemic cells (35.1% of all bone marrow cells) were positive for CD13 (50% of the gated cells), CD33 (78%), CD34 (93%), CD41 (26%), CD56 (35%), and HLA-DR (89%). A diagnosis of transformation to AML with myelodysplasia-related changes was made.

After induction therapy, including idarubicin/cytarabine and daunorubicin/cytarabine regimens, the concentration of bone marrow blasts decreased to 7.2%, but the patient could not achieve complete remission (CR). He then underwent allogeneic peripheral blood stem cell transplantation (PBSCT) from an HLA-identical sibling donor after conditioning with total body irradiation and fludarabine. He achieved complete chimerism, and hematological and cytogenetic CR. However, a bone marrow examination on day 157 revealed 11.4% blasts, indicating hematological relapse. He is now under supportive therapy awaiting a possible second transplantation.

Chromosome analyses of bone marrow cells at the initial diagnosis of MDS and at the diagnosis of AML showed 46,XY,t(3;12)(q26.2;p13)[11]/46,XY[9] and

46,XY,t(3;12)(q26.2;p13)[7]/46,sl,del(5)(q?)[13], respectively (Figure 1B). The karyotypes before PBSCT and at relapse after PBSCT were 46,XY,t(3;12)(q26.2;p13)[14]/46,sl,del(7)(q?)[1]/46,XY[5] and 46,XY,t(3;12)(q26.2;p13),del(7)(q?)[18]/46,sl,-Y,add(2)(q31),del(6)(q?),add(10)(q22)x2,add(21)(q22),+mar1[2], respectively. Spectral karyotyping (SKY) confirmed both der(3)t(3;12)(q26.2;p13) and der(12)t(3;12)(q26.2;p13) (Figure 1C). Fluorescence *in situ* hybridization (FISH) detected the split *ETV6* and *EVI1* signals (data not shown).

We next performed reverse transcription–polymerase chain reaction (RT–PCR) for the possible detection of *ETV6/MECOM* fusion transcripts. We designed a forward primer, ETV6-F2 (from *ETV6* exon 2, 5'-TCAGGATGGAGGAAGACTCG -3', cDNA positions 396–415 according to NCBI reference sequence NM_001987.4), and a reverse primer, EVI1-R3 (from *MECOM* exon 3, 5'-ACTTGAGCCAGCTTCCAAC-3', cDNA positions 833–851 according to NM_004991.3). Two PCR products of 453 bp and 115 bp were successfully amplified in the patient's bone marrow cells at diagnosis of AML (Figure 1D, lane 2). Nucleotide sequencing of the major PCR product revealed that the *ETV6* exon 2 was in-frame fused to the *MDS1* exon 2 (Figure 1E), whereas the sequences of the minor PCR product were out-of-frame fusion between the *ETV6* exon 2 and the *EVI1* exon 2 (Figure 1F). Similar PCR products were also amplified in the patient's peripheral blood mononuclear cells at the MDS stage (Figure 1D, lane 1).

We also established a real-time quantitative PCR (RT–qPCR) strategy to monitor minimal residual disease (MRD) after chemotherapy. RT-qPCR for *ETV6/MDS1/EVI1* was performed using a forward ETV6-F2 primer, reverse MDS1-EVI1-R primer (5'-AGAGCATGGCTCTTGAATATTG-3', cDNA positions 526-547), MDS1-EVI1 probe (5'-[FAM]-TGGCTACTCCATCTGCATCTGGCATTTCTT-[TAM]-3'), TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA), and QuantStudio 12K Flex

(Applied Biosystems, Foster City, CA). The reaction mixture consisted of 4μl cDNA template (200ng of RNA equivalent), 0.25mM of each probe, and 0.5mM of each primer. Cycling parameters were 95°C for 20 sec to activate DNA polymerase, then 40 cycles of 95°C for 1 sec and 60°C for 20 sec. A forward primer ETV6-F3 (5'-GATGGAGGAAGACTCGATCC-3', cDNA positions 400–419), reverse EVI1-R3 primer, and EVI1 probe (5'-[FAM]-GGTTGACTGGCATCTATGCAGAACTTCACATTGTAAA-[TAM]-3') were used for *ETV6/EVI1*. Each sample was normalized against the corresponding expression of *GAPDH* (Pre-Developed TaqMan Assay Reagent Human GAPDH, Thermo Fisher Scientific). The expression levels of *ETV6/MDS1/EVI1*(/*GAPDH*×10^6) and *ETV6/EVI1*(/*GAPDH*×10^6) were 79,400 and 900 at the MDS stage, and 504,000 and 5,130 at diagnosis of AML, respectively. The expression level of *ETV6/MDS1/EVI1* was approximately 2 log higher than that of *ETV6/EVI1* during the clinical course, and both transcripts varied almost in parallel with disease condition (Figure 1G). They decreased to below the detection limit (<1) after PBSCT, but they rapidly increased on day 129 before hematological relapse (day 157).

We have detected a rare translocation t(3;12)(q26.2;p13), confirmed the coexpression of *ETV6/MDS1/EVI1* and *ETV6/EVI1* fusion transcripts, and monitored MRD in a patient with AML and thrombocytosis. As shown in Table 1, six cases of hematological malignancies with the *ETV6/MDS1/EVI1* and/or *ETV6/EVI1* fusion have been reported: two presented with only *ETV6/MDS1/EVI1*, three presented with only *ETV6/EVI1*, and one presented with both [7-11]. Thus, the present case is the second one with both fusion transcripts to be identified, although the influence of this molecular heterogeneity on transforming activity and disease phenotype remains to be elucidated. Although the difference of the breakpoints around *EVI1* was shown by FISH [1,4], the structures of both transcripts were identical in all

cases: *ETV6* exon 2/*MDS1* exon 2/*EVI1* exon 2 and *ETV6* exon 2/*EVI1* exon 2. In case 1, both transcripts were detected at transformation of atypical CML, whereas they were not examined at the chronic phase. In the present case, we performed serial RT-PCR and showed that both transcripts, rather than only one, were already expressed at the MDS stage as well as at diagnosis of AML. Thus, coexpression of *ETV6/MDS1/EVI1* and *ETV6/EVI1* may be implicated in the development of MDS with t(3;12)(q26.2;p13) at an early step rather than the progression to AML.

We first successfully monitored MRD for *ETV6/MECOM* fusion transcripts by RT-qPCR and revealed that the expression level of *ETV6/MDS1/EVI1* was approximately 2 log higher than that of *ETV6/EVI1* during the clinical course. These results indicated that the *ETV6/EVI1* may be a minor splice variant of *ETV6/MDS1/EVI1* instead of being derived from a distinct clone with a different breakpoint, as suggested previously [7]. It is probable that the genomic 3q26.2 breakpoint was located within the *MDS1* intron 1. Interestingly, in case 4 of Table 1, the *ETV6/EVI1* appeared following t(9;22)(q34;q11.2) before blast crisis of CML [9]. In case 5, trisomy 8 was found in the stem line and t(3;12) leading to the *ETV6/EVI1* emerged as the secondary abnormality [10], thus, in cases harboring *ETV6/EVI1* alone, this fusion could be an additional genetic event. In fact, the ETV6/MDS1/EVI1 fusion protein is in-frame, whereas ETV6/EVI1 is an out-of-frame fusion despite the open reading frame of EVI1 remains intact [3]. Therefore, together with data in the present case, it is suggested that the *ETV6/MDS1/EVI1* but not *ETV6/EVI1* plays a crucial role in the pathogenesis of MDS/AML with t(3;12).

Another noticeable hematological finding was thrombocytosis at the diagnosis of AML, although thrombocytopenia was the initial manifestation at the diagnosis of MDS. AML with inv(3)(q21.3q26.2)/t(3;3)(q21.3;q26.2) and *MECOM* rearrangements frequently demonstrates normal or elevated platelet counts [1], whereas MDS/AML with t(3;12)(q26;p13)

usually displays normal or low platelet count [3,5], and t(3;12) with thrombocytosis seems to be rare [12]. There is no other case with thrombocytosis in Table 1, and it is unlikely that the platelet count was correlated with the type of fusion transcript. In the present case, both *ETV6/MDS1/EVI1* and *ETV6/EVI1* fusion transcripts were similarly expressed at diagnoses of MDS and AML. Thus, instead of fusion transcripts, it is possible that an additional cytogenetic abnormality del(5)(q?), found at diagnosis of AML, induced thrombocytosis. After chemotherapy, del(7)(q?) but not del(5)(q?) appeared as an additional anomaly, and thrombocytosis was not observed. Regarding the association between del(5q) and thrombocytosis, the 5q- syndrome is characterized by macrocytic anemia, a normal or high platelet count, and hypolobulated micromegakaryocytes [13]. It has been suggested that haploinsufficiency of the miR-145/miR-146a at 5q32-33 may contribute to the thrombocytosis seen in the 5q- syndrome [13]. This mechanism might be applicable to the present case. Namely, an additional del(5)(q?) concomitant with t(3;12) might be associated with thrombocytosis. Two other cases of AML with t(3;12)(q26;p13) and del(5)(q) were reported [14], but their platelet counts were unclear. Further studies are required to clarify the correlation between t(3;12)(q26;p13) with del(5q) and thrombocytosis.

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Figure legends

Figure 1.

(A) Bone marrow smears at diagnosis of AML show myeloblasts with fine nuclear chromatin, nucleoli, and basophilic cytoplasm (left), mononuclear micromegakaryocytes (middle), and a giant platelet (right) (May-Grünwald-Giemsa staining, ×1000).

(B) G-banded karyotype of bone marrow cells at diagnosis of AML: 46,XY,t(3;12)(q26.2;p13),del(5)(q?). Arrows indicate rearranged chromosomes.

(C) Spectral karyotyping of metaphase spreads after spectrum-based classification (left side, reverse DAPI; right side, SKY). Only chromosomes 3 and 12 are shown. Two derivative chromosomes, der(3)t(3;12)(q26.2;p13) and der(12)t(3;12)(q26.2;p13), are confirmed. Arrows indicate rearranged chromosomes.

(D) Detection of the *ETV6/MDS1/EVI1* and *ETV6/EVI1* fusion transcripts by RT-PCR analysis. Lane M, DNA of a 100-bp ladder as a size marker; lane 1, peripheral blood mononuclear cells of the patient at the MDS stage (20 months after the initial diagnosis); lane 2, bone marrow cells of the patient at diagnosis of AML; lane 3, normal bone marrow cells (negative control). Two PCR products of 453 bp and 115 bp are similarly amplified at diagnosis of MDS and AML.

(E) Nucleotide and amino acid sequences surrounding the junction of the major PCR product (453 bp). The *ETV6* exon 2 is in-frame fused with the *MDS1* exon 2 (*MECOM* exon 2). The vertical arrow indicates the breakpoint.

(F) Nucleotide and amino acid sequences surrounding the junction of the minor PCR product (115 bp). The *ETV6* exon 2 is out-of-frame fused with the *EVI1* exon 2 (*MECOM* exon 3). The vertical arrow indicates the breakpoint.

(G) Serial RT–qPCR analyses for the *ETV6/MDS1/EVI1* and *ETV6/EVI1* fusion transcripts. Total RNAs were isolated from peripheral blood mononuclear cells at the MDS stage (20

and 24 months after initial diagnosis), and sequentially from bone marrow cells after diagnosis of AML (as of 60 months after diagnosis). The almost parallel correlation between the logarithm of *ETV6/MDS1/EVI1* (*/GAPDH* $\times 10^6$) and that of *ETV6/EVI1* (*/GAPDH* $\times 10^6$) was observed during the clinical course. The expression level of *ETV6/MDS1/EVI1* was approximately 2 log (1.80 to 1.99 log) higher than that of *ETV6/EVI1* before PBSCT. Both expression levels decreased to below the detection limit after PBSCT, but they rapidly increased before hematological relapse. The *ETV6/MDS1/EVI1* level was more than 2 log (2.01 to 2.33 log) higher than the *ETV6/EVI1* level at the relapse after PBSCT

Fig. 1.

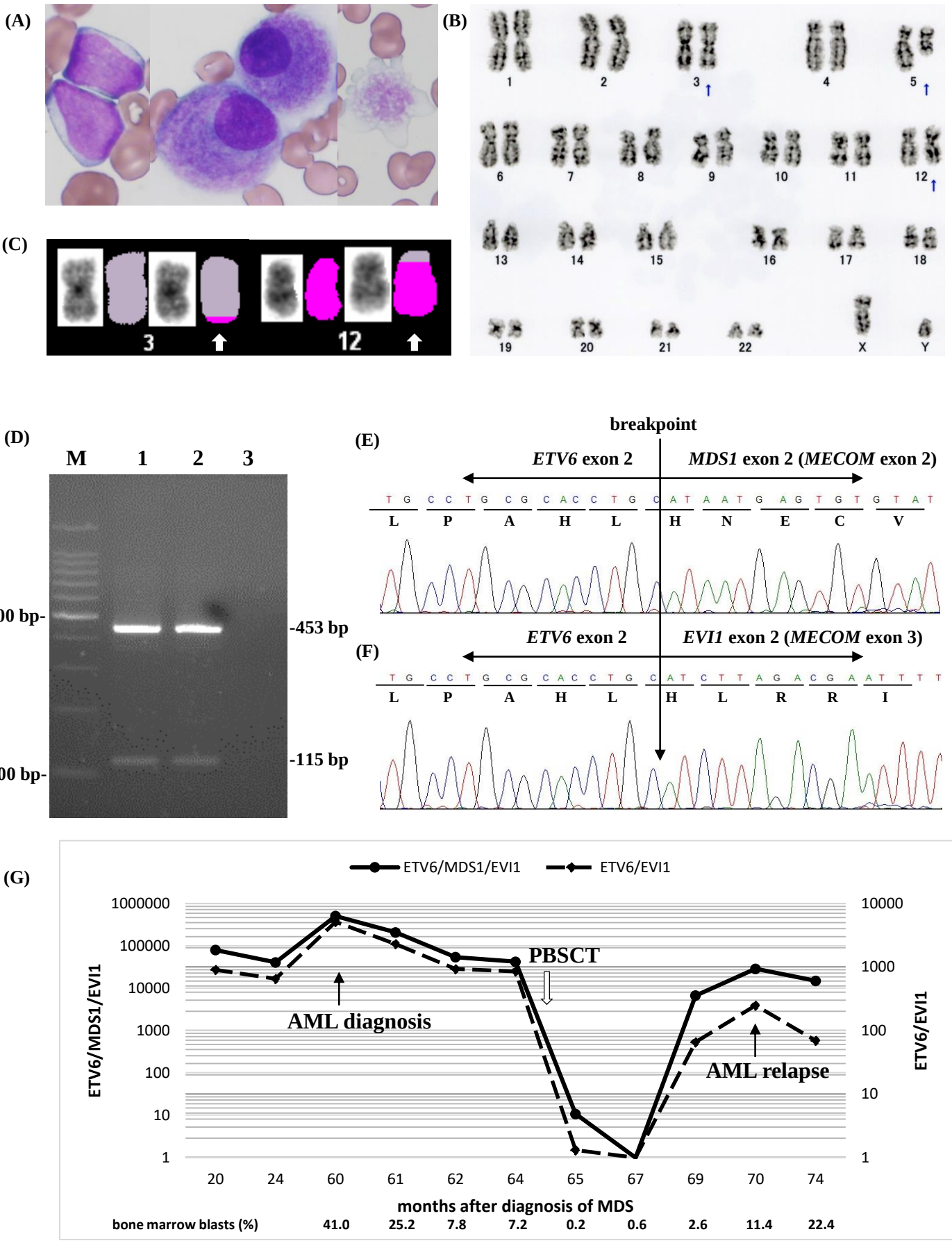
(A)
(B)
(C)
3
12
(D)
M
1
2
3
500 bp-
100 bp-
-453 bp
-115 bp
(E)
breakpoint
ETV6 exon 2
MDS1 exon 2 (MECOM exon 2)
T G C C T G C G C A C C T G C A T A A T G A G T G T G T A T
L P A H L H N E C V
(F)
ETV6 exon 2
EVI1 exon 2 (MECOM exon 3)
T G C C T G C G C A C C T G C A T C T T A G A C G A A T T T T
L P A H L H L R R I
(G)
ETV6/MDS1/EVI1
ETV6/EVI1
1000000
100000
10000
1000
100
10
1
10000
1000
100
10
1
ETV6/MDS1/EVI1
ETV6/EVI1
AML diagnosis
PBSCT
AML relapse
20 24 60 61 62 64 65 67 69 70 74
months after diagnosis of MDS
bone marrow blasts (%) 41.0 25.2 7.8 7.2 0.2 0.6 2.6 11.4 22.4

Table 1. Reported cases of hematological malignancies associated with the *ETV6/MDS1/EVI1* and/or *ETV6/EVI1* fusion transcripts

Case No.	*Age/ Sex*	*Diagnosis*	*Karyotypes*	*ETV6/MDS1/EVI1 fusion transcript*	*ETV6/EVI1 fusion transcript*	*Plt ($x10^9$/L)*	*References*
1	26/M	atypical CML in tansformtion	46,XY,**t(3;12)(q26;p13)**,t(9;15;12)(p21;q15;p13)[6]	exon 2/exon 2*/exon 2*	exon 2/exon 2	37	Peeters et al., 1997 [7]
2	33/F	MDS RAEB-t	46,XX,**t(3;12)(q26;p13)**,del(7)(q22)[31]	exon 2/exon 2/exon 2	ND	311	Peeters et al., 1997 [7]
3	49/F	AML M0	46,XX,**t(3;12)(q26;p13)**,add(7)(q11)	exon 2/exon 2/exon 2	ND	46	Nishimura et al., 2000 [8]
4	26/M	CML-BC	46,XY,**t(3;12)(q26;p13)**,t(9;22)(q34;q11)	ND	exon 2/exon 2	NA	Nakamura et al., 2002 [9]
5	63/F	AML M4	47,XX,+8[2]/47,sl,**t(3;12)(q26;p13)**[18]	ND	exon 2/exon 2	1.32	Al-Achkar et al., 2014 [10]
6	60/M	t-AML	45,XY,**t(3;12)(q26;p13)**,-7[16]	ND	exon 2/exon 2	68	Kim et al., 2017 [11]
7	21/M	MDS-SLD	46,XY,**t(3;12)(q26.2;p13)**[11]/46,XY[9]	exon 2/exon 2/exon 2	exon 2/exon 2	86	present case
		AML-MRC	46,XY,**t(3;12)(q26.2;q13)**[7]/46,sl,del(5)(q?)[13]	exon 2/exon 2/exon 2	exon 2/exon 2	605	

Abbreviations: F, female; M, male; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; RAEB-t, refractory anemia with excess blasts in transformation; AML, acute myeloid leukemia; BC, blastic crisis; t-AML, therapy-related AML; SLD, single lineage dysplasia; MRC, myelodysplasia-related changes; ND, not detected; NA, not available; Plt, platelet count. The t(3;12)(q26;p13) is depicted in bold letters. **MDS1* exon 2 and *EVI1* exon 2 corresponds to *MECOM* exon 2 and *MECOM* exon 3, respectively.