EVI1 overexpression in t(3;17) positive myeloid malignancies results from juxtaposition of *EVI1* to the *MSI2* locus at 17q22

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ABSTRACT

Chromosomal translocations involving the *EVI1* locus are a recurrent finding in myeloid leukemia and are associated with poor prognosis. In this study, we performed a detailed molecular characterization of the recurrent translocation t(3;17)(q26;q22) in 13 hematologic malignancies. The *EVI1* gene locus was rearranged in all 13 patients and was associated with *EVI1* overexpression. In 9 out of 13 patients, the 17q breakpoints clustered in a 250 kb region on band 17q22 encompassing the *MSI2* (*musashi homologue 2*) gene. Expression analyses failed to demonstrate ectopic *MSI2* expression or the presence of an *MSI2/EVI1* fusion gene. In conclusion, we show for the first time that the t(3;17) is indeed a recurrent chromosomal aberration in myeloid malignancies. In keeping with findings in other recurrent 3q26 rearrangements, overexpression of the *EVI1* gene appears to be the major contributor to leukemogenesis in patients with a t(3;17).

Key words: *EVI1*, *MSI2*, myeloid malignancies, t(3;17), FISH.

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Introduction

The Ecotropic Viral Integration site 1 (*EVI1*) gene is located at chromosome band 3q26.2 and was identified as a proto-oncogene by retroviral integration assays in mice.[1] Rearrangements of the *EVI1* locus are found in acute myeloid leukemias (AML), myelodysplastic syndromes (MDS) and chronic myeloid leukemias (CML). *EVI1* gene rearrangements account for approximately 5% of cytogenetic abnormalities in these disease entities.[2] Patients with an *EVI1* rearrangement have distinct clinical features, such as marked hyperplasia with dysplasia of the megakaryocytes[3] and, in some cases, hyperthrombocytosis.[4]

These 3q26 chromosomal aberrations confer an adverse prognosis and contribute to ectopic expression of either full length or truncated *EVI1* transcripts, or to the formation of *EVI1* fusion genes.[2] Common recurrent rearrangements affecting the 3q26 locus include the inv(3)(q21q26) and the translocation t(3;3)(q21;q26), in which *EVI1* overexpression is caused by juxtaposition of the *EVI1* gene to enhancer elements of the Ribophorin (RPN) gene at 3q21 (5). *EVI1* activation in the translocations t(3;12)(q26;p13) and t(3;21)(q26;q22) is due to generation of the fusion genes *ETV6/EVI1* and *RUNX1/EVI1* respectively.[6,7]

In addition to these well-characterized rearrangements, the *EVI1* locus is also involved in rare 3q26 aberrations such

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as the t(3;17)(q26;q22),[8] the t(2;3)(p21~22;q26)[9,10] and the t(3;6)(q26;q25).[11] For these *EVI1* rearrangements, the true recurrent nature and the partner chromosomes involved, have not been analyzed in detail. Therefore, we performed an in depth characterization of the 17q breakpoints in 13 hematologic malignancies with a t(3;17).

Design and Methods

Patients and cell lines

In this multicenter retrospective study 13 leukemias were included according to the following criteria; presence of a hematologic malignancy with a t(3;17) and 3q26 rearrangement. Karyotyping of diagnostic samples was performed according to standard procedures. Several myeloid leukemia cell lines (K562 and U937) and *EVI1* rearranged cell lines (Kasumi-3 and UCSD-AML1) were included as positive controls for *EVI1* overexpression.[12-15] The study was approved by the ethics committee of the Ghent University Hospital (2003/273). Patients' characteristics and karyotypes are described in Table 1.

Fluorescence in situ *hybridization*

Dual-color fluorescence *in situ* hybridization (FISH) with a dedicated *EVI1* probe set (RP11-362K14, RP11-82C9 and RP11-694D5) was performed on fixed nuclei and/or metaphases of patients and cell lines.[8] To characterize the 17q breakpoints, eight different 17q BAC/PAC probes over a region of 34.3 Mb on 17q were selected from the UCSC (*http://genome.ucsc.edu*) or Ensembl (*http://ensembl.org*) databases. The 17q and EVI1 probes were all obtained from the Sanger Wellcome Trust Institute, Hinxton, Cambridge (United Kingdom). Position of the probes used and location of translocation breakpoints is described in Figures 1 A–B.

Real-time quantitative RT-PCR

Total RNA was extracted from total bone marrow samples or bone marrow leukocytes from 9 of 13 t(3;17) samples, from 4 myeloid cell lines, from bone marrow of 3 patients without a hematologic disorder and from 2 stem cell ($CD34^+$) fractions, using the miRNeasy kit (Qiagen, Belgium) according to the manufacturer's recommendations. The cell lines and $CD34^+$ cells served as positive controls for the study of *EVI1* expression as well as for *MSI2* expression. cDNA was prepared from 2 µg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Belgium) according to the manufacturer's descriptions.

Real-time quantitative RT-PCR (qRT-PCR) for the *EVI1* (exon 1b-2), *cEVI1* (exon 8-9, primers located at the 3' end of the *EVI1* gene to detect possible 5' tran-

Table 1 Patients' characteristics, diagnosis, sex, age at diagnosis, G-banded karyotype.

Case	*Diagnosis*[†]	*Percentage of blasts in t(3;17) sample*	*Age at diagnosis (years)*	*Sex*[‡]	*Karyotype*	*EVI1*	*Treatment*	*Survival*
1	MDS	8	66	F	46,XX,t(3;17)(q26;q22)[12]	-	Supportive transfusions	7 years
2	CML-BC	48	25	M	46,XY,t(3;17)(q26;q22),t(9;22)(q34;q11.2)[18]	**	AraC	10 months
3	CML-BC	45	69	M	46,XY,t(3;17)(q26;q22),t(9;22)(q34;q11.2), t(12;13)(p12;q13)[16]/47,sl,+8[2]	**	Hydrea	4 months
4	AML	22	6	M	45,XY,t(3;17)(q26;q22),-7[18]	+	AraC/VP16/M	6 months
5	MDS	7	58	M	47,XY,+3,del(3)(q25q27),t(3;17)(q26;q12), del(6)(p23),-20,-21,+2mar[8]	–	AraC/VP16/D	15 months
6	CML-BC	59	65	M	46,XY,t(9;22)(q34;q11.2)[2]/ 46,sl,t(3;17)(q26;q22)[18]	+	Hydrea/6-M	4 months
7	AML	29	64	F	46,XX,t(3;17)(q26;q22),t(9;22)(q34;q11.2)[5]/ 46,XX[7]	+	**	**
8	AML	15	73	F	45,XX,t(3;17)(q26;q22),-7[6]	–	6-T/G	alive (2 years)¶
9	AML	29	66	F	46,XX,t(3;17)(q26;q22)[10]	**	AraC/D	**
10	CML-BC	45	57	M	50,XY,t(3;9;22;17)(q26;q34;q11;q22), +8,+der(9)t(3;9;22;17), +10,+12,del(16)(q23)[15]	+	AraC/D	9 months
11	MDS	15	42	M	46,XY,t(3;17)(q26;q22)[21]/ 46,sl,der(16)t(1;16)(q12;q12)[2]	–	allo SC	alive (3 years) ¶
12	AML	23	25	M	45,XY,t(3;17)(q26;q22),-7[2]/ 46,sl,+12[10]	+	AraC/D	7 months
13	AML	47	63	M	44,XY,t(2;3;17)(p21;q26;q22), der(7)t(7;15)(p14;q15),der(10)t(10;16) (q26;q12~q13),-15,-16[25]	**	AraC/I/CCNU/ Hydrea	15 months

*[†]MDS: myelodysplastic syndrome; CML: chronic myeloid leukemia; CML-BC: CML in blast crisis and AML: acute myeloid leukemia (according to FAB classification) [‡]M: male and F: female. AraC : 1-β-D-arabinofuranosylcytosine; D: daunorubicin, VP16: etoposide; M: mitoxantrone; I: idarubicine; H: hydroxyurea; allo SC: allogenic stem cell transplantation, 6-T = 6-thioguanine, G = gemtuzumab ozogamicin, 6-M = 6-mercaptopurine and CCNU = lomustine ¶ Survival time at publication ** No data available +Positive for EVI1 overexpression with qRT-PCR. - Negative for EVI1 overexpression with qRT-PCR.*

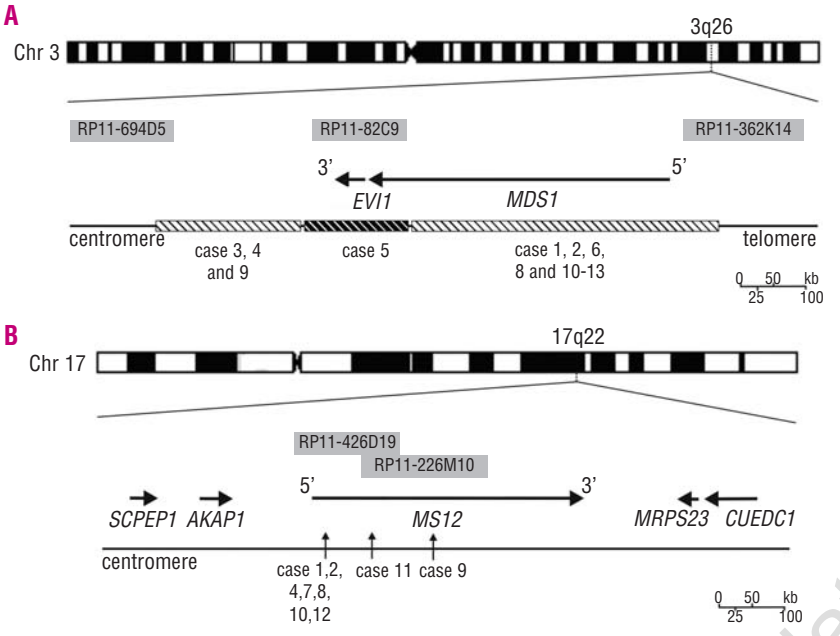

Figure 1. Schematic overview of the location of the 17q and 3q probes and breakpoints. (A). Physical map of the 3q26.2 probes used. The locations of the *EVI1* breakpoints in the 13 t(3;17) patients are indicated by shaded boxes. (B). Location of the 17q22 breakpoint for 9 out of 13 patients. Vertical arrows indicate location of the breakpoints within the *MSI2* gene.

script variants),[16] *MDS1/EVI1* (*data not shown*) and MSI2 (primer pairs for exon 3 and 10) transcripts was performed as previously described.[8,17] For normalization three housekeeping genes (*RPL13A*, *YWHAZ* and *HPRT1*) were selected in view of their stability in bone marrow samples as analyzed using the Genorm software.[17] The *EVI1* overexpressing cell line Kasumi-3 carrying a t(3;7)(q26;q22) translocation[12] was used as a positive control.

RT-PCR

To investigate the possibility of *MSI2/EVI1* fusion gene formation, RT-PCR was performed. Based on the known *EVI1* breakpoint position in fusion genes and the location of the *MSI2* 17q breakpoints, primers for the second exon of *EVI1* and the second exon of *MSI2* were selected. For PCR analysis, the following touchdown program was used: an initial denaturation step at 94°C for 2 min, 12 cycles of 20 sec at 94°C, 15 sec at the initial annealing temperature (Ta) 62°C (Ta-1°C for each cycle), 1 min at 72°C followed by 24 cycles of 40 sec at 94°C, 40 sec at 50°C, 30 sec at 72 °C and a final extension step of 4 min at 72°C.

Results and Discussion

FISH analysis demonstrated a rearrangement of the *EVI1* locus in all samples. For 9 out of 13 leukemic samples, the 3q26 breakpoint was located 5' (telomeric) of the *EVI1* gene which is in keeping with previous observations of 3q26 translocation breakpoint positions.[8] In 3 out of 13 samples, the *EVI1* breakpoint was located 3' (centromeric) of the *EVI1* gene and in case 5 the *EVI1* breakpoint was located within the region corresponding to the RP11-82C9 probe and could thus be located either 5', 3' or within the *EVI1* gene (Figure 1A).

In 9 of 13 patients the 17q breakpoint clustered in a 250 kb region on 17q22 harboring the *MSI2* gene (Figure 1B). For the remaining 3 cases, unique breakpoints were detected in BAC clones RP11-386F9 (patient #5, 17q11.2), RP11-1094H24 (patient #3, 17q21.33) and in the PAC clone RP5-1171I10 (patient #6, 17q22), respectively (*data not shown*). For patient #13, the 17q breakpoint overlapping clone could not be determined.

MSI2 plays a role in post-transcriptional gene regulation and in maintaining stem cell status,[18] and involvement of *MSI2* in myeloid leukemias has previously been reported in 2 CML patients, one of which carried a *MSI2/HOXA9* fusion gene.[19] In our patient series however, no *MSI2/EVI1* fusion gene could be detected in RT-PCR analysis. Given the opposite orientation of *MSI2* and *EVI1* and the lack of karyotypic evidence for complex rearrangements, this result was indeed anticipated.[20] Further study of a possible *MSI2/EVI1* fusion transcript was complicated by limited availability of patient sample. *MSI2* rearrangements have also been linked to CML and myeloproliferative disorder disease progression.[19,21,22] Interestingly, 2 out of 9 patients with a *MSI2* breakpoint were CML-BC patients.

Frequently observed secondary karyotypic changes in CML include +8, i(17q), +19 and +Ph, but in some occasions recurrent translocations and 3q26 rearrangements are reported.[23] We postulate that the t(3;17) involving *MSI2* is associated with CML as a secondary aberration and could serve as a progression marker.

Real-time quantitative PCR with *EVI1* and *cEVI1* primers indicated that 5 out of 9 patients displayed ectopic *EVI1* expression (*data not shown*). In the remaining 4 patients, no ectopic *EVI1* expression could be detected. In a previous study, we showed that the majority of hematologic malignancies displaying a

rearrangement in the vicinity of *EVI1* show ectopic *EVI1* expression.[8] Therefore, we hypothesize that in these 4 patients *EVI1* expression could not be detected because of the low blast counts in these samples. Overexpression of the *EVI1* oncogene conveys a poor prognosis in AML and CML-BC.[24] In our cohort, patients without detectable *EVI1* overexpression had prolonged survival compared to the patients with *EVI1* overexpression (Table 1). This enhanced prognosis could in part be explained by the absence of a high blast count which is indicative for an early stage of the disease. Therefore the patient's blast count might have to be taken into account when addressing prognosis.

The exact cause of *EVI1* overexpression is not known, but in the inv(3)(q21q26) and the t(3;3)(q21;q26) *EVI1* is juxtaposed to the enhancer elements of the Ribophorin gene on chromosome band 3q21.[5] Possibly, *EVI1* overexpression in t(3;17) leukemias is driven by enhancer elements located in the vicinity of the *MSI2* locus.

We found no significant differences in *MSI2* expression between *MSI2* rearranged and *MSI2* non-rearranged t(3;13) hematologic malignancies (*data not shown*). Expression of *MSI2* in patients with a 17q breakpoint in *MSI2* is not straightforward. Due to the translocation, a part of the *MSI2* gene is separated from its promoter. Therefore, we postulate that the observed expression is due to residual expression from the normal allele.

No *MSI2/EVI1* fusion gene could be detected, and no significant *MSI2* expression differences were found between *MSI2* rearranged and *MSI2* non-rearranged patients. Therefore, we have no evidence of a transforming role of *MSI2*. Given the expression of *MSI2* in $CD34^+$ cells (*data not shown*) it is feasible that the gene is located in a region with open chromatin within the genome. As these regions are more susceptible to double strand DNA breaks, this could lead to increased vulnerability of the *MSI2* locus in chromosomal aberrations. Rearrangement and consecutive overexpression of *EVI1* seem to be the main contributor to tumorigenesis in t(3;17) patients.

In conclusion, we confirm the t(3;17) as a recurrent chromosomal aberration in myeloid malignancies, consistently involving *EVI1*. We found that a distinct subgroup (9 out of 13) shares a recurrent 17q breakpoint region implicating the *MSI2* gene, and postulate that overexpression of the *EVI1* gene is the major contributor to leukemogenesis in patients with a t(3;17).

Authorship and Disclosures

ADW: performed FISH and expression analysis, drafted the manuscript; FS: helped drafting the manuscript; BC: helped with the RT-PCR; NVR: helped drafting the manuscript; NY: helped with FISH experiments; BV: helped with drafting the manuscript; BDM, YB, LN, DS, EL, SS, CB, PV, AH, ND: supplied patient samples; ADP: helped drafting the manuscript; BP: helped drafting the manuscript.

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