RNA sequencing reveals a unique fusion of the lysine (K)-specific methyltransferase 2A and smooth muscle myosin heavy chain 11 in myelodysplastic syndrome and acute myeloid leukemia

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are heterogeneous malignancies characterized by a variety of acquired genetic abnormalities and variable response to treatment.[1,2] In the last decade, a number of novel molecular genetic abnormalities have been revealed in MDS and AML by applying novel genome-wide technologies, such as massively parallel sequencing.[2,3] The different recurrent genetic aberrations shed light on possible mechanisms involved in leukemogenesis and refine risk stratification of both diseases.[1] Although recurrence of aberrations in MDS and AML is the major guide to reveal general mechanisms regarding leukemogenesis, unique abnormalities can also be highly informative. Here we describe a unique fusion of the lysine (K)-specific methyltransferase 2A (*KMT2A*) gene [mixed-lineage leukemia gene (*MLL*)], located on chromosome 11q23, and the gene encoding smooth muscle myosin heavy chain 11 (*MYH11*), located on chromosome 16p13, in a patient with MDS and subsequently AML, both harboring the cryptic translocation t(11;16). *KMT2A* and *MYH11* are involved in recurrent translocations in AML, but fusions of these two genes have never been demonstrated.

KMT2A is a transcriptional activator, which regulates gene expression, including *HOX* genes, by methylation of histone H3 lysine 4 (H3K4).[4] The *KMT2A* gene on 11q23 is involved in translocations in approximately 5% of adult AML cases[4] and more than 70 translocation partners of *KMT2A* have been described.[5] The majority of *KMT2A* fusions incorporate the N-terminal portion of KMT2A, containing three short AT–hook motifs, two speckled nuclear localization sites, and a transcriptional repression domain (Figure 1B).[4,5] Leukemic *KMT2A* fusions impair H3K4 methylation and transform hematopoietic cells very efficiently.

MYH11 is a subunit of a major contractile protein consisting of two heavy chain subunits and two pairs of non-identical light chain subunits. A pericentric inversion or translocation of chromosome 16 (inv(16)(p13q22) or t(16;16)(p13;q22)), involving the *MYH11* gene, defines a specific subtype of AML characterized by eosinophilia and favorable treatment outcome, and is characteristic for core-binding factor (CBF) leukemias.[1] These chromosome 16 abnormalities result in the fusion of *MYH11* and core-binding factor β (*CBFB*) on 16q22. The resulting fusion transcript *CBFB-MYH11* encodes a protein consisting of the first 133-165 residues of the N-terminus of CBFB and variable C-terminal portions of MYH11. There are two models proposed for CBF-leukemogenesis both based on impairment of the master regulator RUNX1.[6] Briefly, the CBFB-MYH11 fusion protein affects RUNX1 either by sequestering RUNX1 from its target genes or interfering with RUNX1-mediated gene expression by binding of transcriptional repressors to the MYH11 moiety in CBFB-MYH11.[6]

The most frequent *CBFB-MYH11* fusions in adult AML fuses exon 5 of *CBFB* to exon 12 (type A; >85%), exon 8 (type D; <5%) or exon 7 (type E; <5%) of *MYH11*.[7] Several other fusions of *CBFB* and *MYH11* have been demonstrated; however, these are relatively rare (<1%). The variability among *CBFB-MYH11* fusions makes routine detection of this favorable marker in AML by RT-PCR challenging. By applying RT-qPCR aimed for expression of the 3' end of *MYH11*, which is normally not expressed or expressed at a very low level in hematopoietic cells, all AML inv(16) cases are reliably detectable, independent of the type of *CBFB-MYH11*.[8]

Here we describe a 67-year old patient who presented with MDS. After informed consent, bone marrow aspirates and peripheral blood samples were taken at diagnosis and at relapse. Cytological blood smear examination at diagnosis demonstrated a shift to the left in the blood smear with 7% myeloblasts, as confirmed by flow cytometry. Hypogranulated neutrophils were observed, as were Pseudo Pelger-Huet nuclei and occasionally Auer rods. White blood cell count (WBC) was $13.5x10^9$/L. The bone marrow smears were hypercellular with 96% myelopoietic cells and 2% myeloblasts (Figure 1A). Dysmyelopoiesis was seen; however, there was no increased or abnormal eosinophils. The patient was diagnosed as MDS-RAEB II according to the 2008 WHO classification. A diagnosis of MDS was considered based on the elevated WBC and the shift to the left. Patient karyotype at diagnosis was 53,XY,+6,+8,+9,+13,+14,+19,+21[15]. Standard fluorescence *in situ* hybridization (FISH) both on interphase nuclei and metaphases using probes for *KMT2A*/11q23 (break apart), centromere 7 and 8, and probes for 5p15.2 and 5q31 revealed a translocation of *KMT2A*/11q23 to chromosome 16p13. The t(11;16)(q23;p13) was present in 96% of all cells [LSI MLL Dual color break apart probe set (Vysis)]. The patient was treated according to the HOVON43 protocol (*http://www.hovon.nl*). A complete remission was achieved, but the patient relapsed after 41 months. At relapse, the patient was treated with AS602868, a pharmacological inhibitor of the IKK2 kinase, in a phase I trial, but succumbed after progression of the AML. Cytological examination at relapse showed a hypercellular bone marrow, 72% myeloblasts, dysmyelopoiesis and dysmegakaryopoiesis (Figure 1A). Again Auer rods were seen but there was no eosinophilia or abnormal eosinophils. The karyotype was 53,XY,+6,+8,+9,+13,+14,+19,+21[1]/46,XY[19]. FISH demonstrated the t(11;16)(q23;p13) to be present in 90% of all interphases.

All our AML cases are screened with RT-qPCR to detect possible *CBFB-MYH11* fusions. Interestingly, although the patient did not show an inv(16)(p13q22), t(16;16)(p13;q22) by cytogenetic analysis or *CBFB-MYH11* by RT-PCR, *MYH11* was highly expressed at diagnosis and at relapse as shown by RT-qPCR,[8] suggesting that *MYH11* was part of an unknown fusion transcript between 11q23 and 16p13. However, morphologically this case did not show any signs of inv(16)-associated eosinophilia.

To unravel the composition of the *MYH11*-containing mRNA transcript, we performed RNA sequencing (RNASeq) on patient material at diagnosis as part of our ongoing AML research. In brief, total sample RNA was extracted with phenol chloroform and reverse transcribed using Superscript II RT (Life Technologies). The cDNA was sheared with the Covaris device and further processed according to the TruSeq RNA Sample Preparation v.2 Guide (Illumina). Amplified sample libraries were paired-end sequenced (2x36bp) on the HiSeq 2000 system and aligned against the human genome (hg19) using TopHat2.[9] All reads were aligned against genes annotated within the Ensembl database, as provided with the TopHat package. The Integrated Genome Viewer[10] was used for data visualization and assessment of *MYH11* fusion transcripts. The alignment of the RNASeq data confirmed the overexpression of *MYH11*, starting in exon 2 upstream of the ATG-start codon. Interestingly, paired-end sequencing reads of exon 2 of *MYH11* aligned to sequences of exon 8 of *KMT2A*. These results suggested that the t(11;16) resulted in a gene fusion of *KMT2A* (exon 8) and *MYH11* (exon 2)

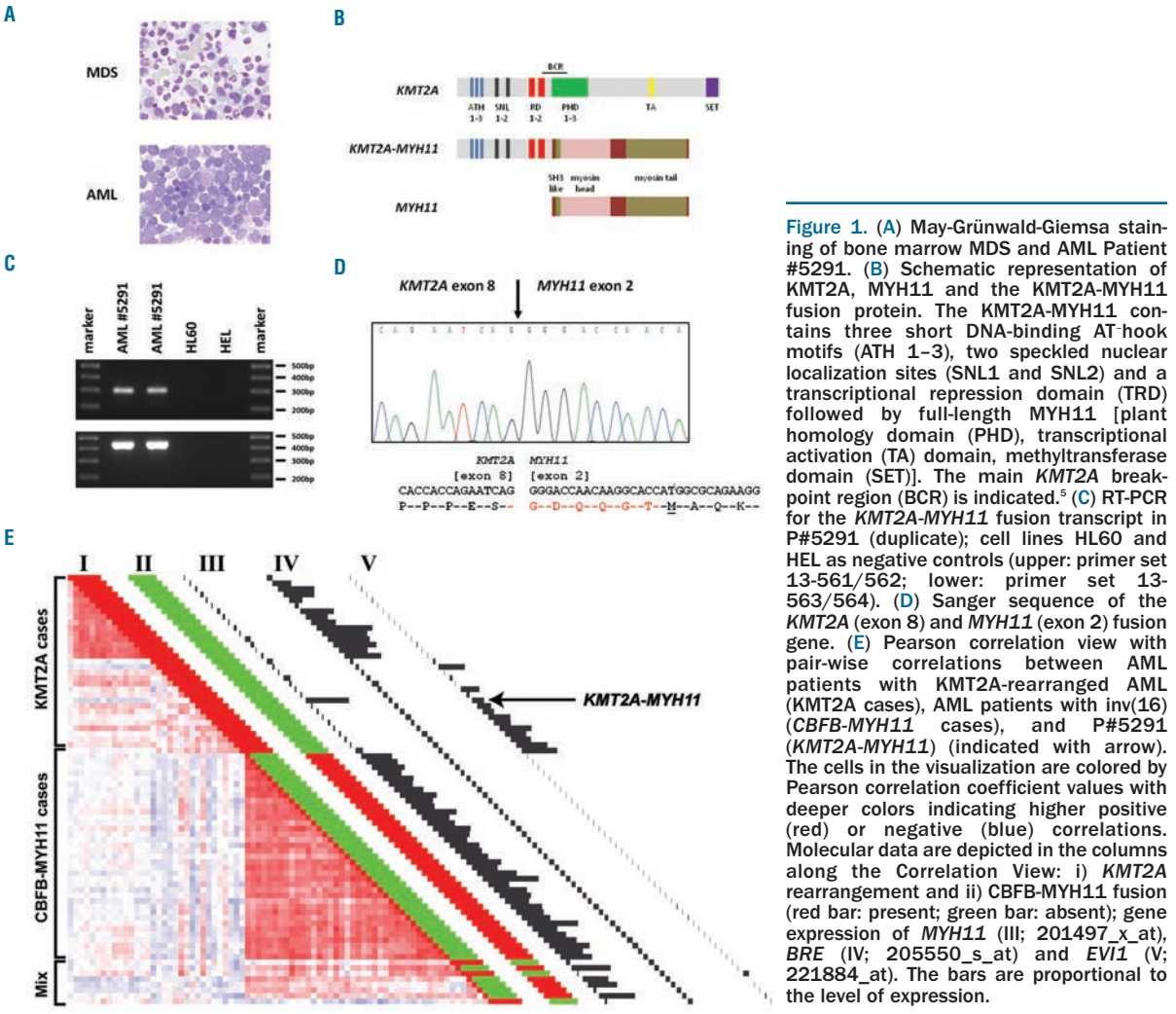

Figure 1. (A) May-Grünwald-Giemsa staining of bone marrow MDS and AML Patient #5291. (B) Schematic representation of KMT2A, MYH11 and the KMT2A-MYH11 fusion protein. The KMT2A-MYH11 contains three short DNA-binding AT-hook motifs (ATH 1–3), two speckled nuclear localization sites (SNL1 and SNL2) and a transcriptional repression domain (TRD) followed by full-length MYH11 [plant homology domain (PHD), transcriptional activation (TA) domain, methyltransferase domain (SET)]. The main *KMT2A* breakpoint region (BCR) is indicated.[5] (C) RT-PCR for the *KMT2A-MYH11* fusion transcript in P#5291 (duplicate); cell lines HL60 and HEL as negative controls (upper: primer set 13-561/562; lower: primer set 13-563/564). (D) Sanger sequence of the *KMT2A* (exon 8) and *MYH11* (exon 2) fusion gene. (E) Pearson correlation view with pair-wise correlations between AML patients with KMT2A-rearranged AML (KMT2A cases), AML patients with inv(16) (*CBFB-MYH11* cases), and P#5291 (*KMT2A-MYH11*) (indicated with arrow). The cells in the visualization are colored by Pearson correlation coefficient values with deeper colors indicating higher positive (red) or negative (blue) correlations. Molecular data are depicted in the columns along the Correlation View: i) *KMT2A* rearrangement and ii) CBFB-MYH11 fusion (red bar: present; green bar: absent); gene expression of *MYH11* (III; 201497_x_at), *BRE* (IV; 205550_s_at) and *EVI1* (V; 221884_at). The bars are proportional to the level of expression.

(Figure 1B). The *KMT2A-MYH11* fusions were confirmed by cDNA amplification using the primer sets 13-561MLL-MYH11 FW1 (*KMT2A* ex7): 5'-TTCCAGGAAGTCAAG-CAAGC-3' and 13-562MLL-MYH11 RV1 (*MYH11* ex2): 5'-CTCGAAGCCCTGCTTCTC-3' (amplicon:298bp) or 13-563MLL-MYH11 FW2 (*KMT2A* ex7): 5'-CCGTCGAG-GAAAAGAGTGAA-3' en 13-564MLL-MYH11 RV2 (*MYH11* ex2): 5'-CGTGACCTTCTTGCCATTCT-3' (amplicon:443bp) (0.25mM dNTP,15pmol primers,2mM $MgCl_2$, Taq polymerase and 1 x buffer (Life Technologies). Cycling conditions were: one cycle of 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and one cycle of 7 min at 72°C. PCR amplification with both primer sets resulted in products with the expected size (Figure 1C). These PCR products were sequenced by using forward and reverse primers on the ABI PRISM3100 genetic analyzer (Applied Biosytems Life Technologies). Sanger sequencing confirmed the *KMT2A-MYH11* fusion transcript encoding an in-frame KMT2A-MYH11 fusion (Figure 1D). The *KMT2A-MYH11* fusion was demonstrated to be present at relapse (*data not shown*). Lack of high-quality protein lysates prevented detection of the KMT2A-MYH11 fusion protein in the patient's MDS and AML phase.

Gene expression analyses demonstrated that the *KMT2A-MYH11* AML did not show any correlation with *CBFB-MYH11* AML (Figure 1E).[11,12] Interestingly, however, based on gene expression, the *KMT2A-MYH11* AML grouped together with *MLL*-rearranged AML (Figure 1E). More specifically, the *KMT2A-MYH11* AML clustered with *MLL*-rearranged AML with high *EVI1* expression[13] instead of high *BRE* expression.[14]

Extensive analyses of the RNASeq data demonstrated a mutation in a well-known AML- and MDS-related gene, a non-synonymous mutation in the splicing factor gene *U2AF1* (exon2:c.C101T:p.S34F). This mutation in *U2AF1* has been confirmed by Sanger sequencing. Whether this mutation is somatic or germline remains to be elucidated.

In the past, several t(11;16) patients have been described; however, these cases appear to be rare. In a study of 2 MDS patients with a t(11;16)(q23;p13), a recurrent fusion of the genes encoding KMT2A and CREB-binding protein [CREBBP (CBP)] was demonstrated.[15] A subsequent study of 8 patients revealed that the t(11;16)(q23;p13) occurred exclusively in patients with therapy-related t-AML or t-MDS, i.e. previous treatment with Topo2 inhibitors for a variety of malignancies.[16] Although the breakpoint was not determined in all t(11;16)(q23;p13) cases, it is unlikely that these patients carried a *KMT2A-MYH11* fusion considering the FISH probes used. Furthermore, our patient did not receive any treatment for any prior malignancy.

In this MDS/AML patient, we have revealed a unique fusion of the N-terminal part of KMT2A and the complete

MYH11 protein. This fusion involves two proteins known to be present in chromosomal translocations in highly distinct AML subtypes. All *KMT2A* fusions are subdivided into 4 groups based on the *KMT2A*-fusion partner.[4] MYH11 contains several repeated helical rod domains important for self-dimerization and multimerization in its C-terminus, which also binds transcriptional co-repressors.

The novel KMT2A-MYH11 fusion most probably belongs to group 2 of the MLL-fusion proteins, including SH3GL1/EEN, MLLT4/AF6, GAS7 and AFX1/FOXO4, which all contain oligomerization domains important for transformation. The presence of the t(11;16)(q23;p13) fusion at diagnosis and relapse suggests that KMT2A-MYH11 occurred in the founding clone of the MDS/AML and appears to be essential for this disease.

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