

Molecular studies reveal *MLL-MLLT10/AF10* and *ARID5B-MLL* gene fusions displaced in a case of infantile acute lymphoblastic leukemia with complex karyotype

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Abstract. The present report describes a unique infantile acute lymphoblastic leukemia (ALL) case with cryptic mixed-lineage leukemia (MLL) rearrangements with 11q23 chromosomal translocation. *MLL* break-apart signals were identified by fluorescence *in situ* hybridization, and transcriptome sequencing revealed *MLL*-myeloid/lymphoid or mixed-lineage leukemia; translocated To, 10 (*MLLT10*)/*AF10* fusion transcripts. Analysis also revealed a previously unreported *MLLT10/AF10*-homeobox protein Mohawk (*MKX*) transcript, where the 5' portion of *MLLT10/AF10* at 10p12.31 was fused out-of-frame with the 3' portion of *MKX* at 10p12.1, which is closely located to *MLLT10/AF10*. Furthermore, the reciprocal 3'-*MLL* gene segment was fused in-frame to AT-rich interaction domain (*ARID5B*) at 10q21. Previously, common allelic variants in *ARID5B*, which are directly associated with hematopoietic differentiation and development, have been repeatedly and significantly associated with childhood ALL. The heterozygous genotype in *ARID5B* (RefSNP: rs10821936) increased the risk for leukemia with *MLL*-rearrangement. In particular, single nucleotide polymorphisms of *ARID5B* conferred increased risk for *MLL-MLLT3/AF9*. Based on these findings, the authors propose that while the presence of reciprocal *MLL* alleles has been detected in this patient,

different pathological disease mechanisms may be at play due to individual recombination events.

Introduction

The incidence of translocations in the mixed-lineage leukemia (*MLL*) gene at chromosome band 11q23 is high in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia in infants (1,2). *MLL* forms rearrangements with >60 translocation partner genes (3,4). The most common rearrangements are *AFF1/AF4*, *MLLT1/ENL*, and *MLLT3/AF9*, and the less common are *ELL*, myeloid/lymphoid or mixed-lineage leukemia; translocated To, 10 (*MLLT10*)/*AF10* and *MLLT4/AF6* (3). However, in a significant fraction of patients with leukemia and *MLL* rearrangements, such alterations are absent (3). To address the pathobiology of leukemia with *MLL* rearrangements and determine potential involvement of fusion genes, the authors of the present report previously performed transcriptome sequencing in an infant with ALL and *MLL* rearrangement. The infant was negative for *MLL-AF4*, *MLL-ENL*, *MLL-ELL* and *MLL-AF9* fusion transcripts, and the presence of a *MLL-MLLT10/AF10* fusion transcript was detected (5).

In the present report, a new case of infantile ALL with *MLL-MLLT10/AF10* and 10;11 rearrangements was presented. A chromosomal mechanism leading to *MLL-MLLT10/AF10* fusion and alternative splicing of an *MLL*-exon-8-*MLLT10* fusion genes, resulting in two different isoforms, was described. In addition, it was determined that *MLLT10/AF10*-homeobox protein Mohawk (*MKX*) resulted from an inversion (10p12.1;10p12.31). Furthermore, transcriptome sequencing revealed a separate chromosomal translocation leading to a previously unreported AT-rich interaction domain (*ARID5B-MLL*-positive 10;11 rearrangement in this patient. *ARID5B* polymorphisms are important determinants of childhood ALL susceptibility, and treatment outcomes and contribute to racial disparities in ALL (6). Taken together, these results support the hypothesis of the authors that precise control of *MLL* and *MLLT10/AF10* fusion transcripts is crucial in leukemogenesis.

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Abbreviations: ALL, acute lymphoblastic leukemia; RT-PCR, reverse transcription-polymerase chain reaction; FISH, fluorescence *in situ* hybridization

Key words: acute lymphoblastic leukemia, molecular biology, transcriptome sequencing, *ARID5B*, *MLL-MLLT10/AF10*

Case report

Patient characteristics. A 2-month-old Japanese male infant was admitted to Tokyo University Hospital (Tokyo, Japan) in January 2008. Laboratory tests demonstrated a leukocyte count of $5.44 \times 10^{10}/l$ (normal range, 4.6×10^9 – $18.9 \times 10^9/l$) with 88% blasts, hemoglobin of 9.0 g/dl (normal range, 9.5–13.7 g/dl), and platelet count of $3.9 \times 10^{10}/l$ (normal range, 25×10^{10} – $82 \times 10^{10}/l$). Leukemic cells were cytogenetically characterized as 46, XY, t(2;14)(p11.2;q32), add(11)(q23) (Fig. 1A) and were found to express cluster of differentiation (CD)10 and CD19 by bone marrow biopsy. Analysis with fluorescence *in situ* hybridization using the MLL break-apart probe for the determination of add(11)(q23) revealed the typical split signal (Fig. 1B). Based on the above data, the diagnosis was established as infantile B-precursor ALL with *MLL* rearrangement. The patient achieved complete remission with chemotherapy and received stem cell transplantation. Treatment was well tolerated, and he has been in complete remission for 7 years.

The present study was approved by the Gene Analysis Research Ethics Committee at the University of Tokyo (Tokyo, Japan). Informed consent was obtained from the guardian of the patient.

Paired-end RNA sequencing and identification of fusion genes. High-quality RNA with an RNA integrity number >6.0 from the patient was used to prepare RNA sequencing libraries, according to the TruSeq[®] RNA (Illumina, San Diego, CA, USA) protocol, which were then sequenced on an Illumina HiSeq 2000 device. An in-house pipeline, Genomon-fusion, was used to identify fusion transcripts. All candidate gene fusions that were >2 paired reads were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Sanger sequencing.

RT-PCR and Sanger sequencing. Total RNA (4 μ g) was reverse transcribed to cDNA in a total volume of 33 μ l with random primers using the Ready-To-Go You-Prime First-Strand beads (Pharmacia Biotech; GE Healthcare, Chicago, IL, USA). RT-PCR and Sanger sequencing were performed as previously described (4). In brief, 1 μ l cDNA was used as a template in RT-PCR and the reaction was performed for 35 cycles in a GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), with denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min and a final cycle of 72°C for 7 min. RT-PCR experiment was repeated three times. *MLL-MLLT10/AF10* was amplified using the following forward (F) and reverse (R) primers: MLL F1, 5'-CCTGAGGACTGTGGTGTGTAC-3' and MLLT10/AF10 R1, 5'-CCTGACTGAGAGAAGATCCA GAT-3'. *ARID5B-MLL* was amplified using the following forward and reverse primers: ARID5B F1, 5'-TCGATGCTG AAACGCATCCA-3' and MLL R1, 5'-CACTGCTCTCTT TGCTGTCT-3'. *MLLT10/AF10-MKX* was amplified using the following forward and reverse primers: MLLT10/AF10 F1, 5'-ATGGAAGTTTACAGAGCCTCAG-3' and MKX R1, 5'-TTCGTTTCATGTGGGTCTTGG-3'. Nucleotide sequences of PCR products and, if necessary, subcloned PCR products were analyzed as described previously (4).

Detection of fusion transcripts. To identify high-confidence fusion transcripts, an in-house pipeline for RNA sequencing data analysis, Genomon-fusion, was used for analysis of RNA sequencing data from the patient bone marrow cells. A total of 49 fusion transcripts supported by discordant read pairs as well as one perfectly matched junction-spanning read, with the other end of the read-pair mapping to either of the fusion gene partners, were identified.

To focus on fusion transcripts identified in infantile leukemia, from the initial list of 49 fusion transcripts, two in-frame fusion transcripts (*MLL-MLLT10/AF10* and *ARID5B-MLL*) and one out-of-frame fusion transcript (*MLLT10/AF10-MKX*) that were supported by >10 junction-spanning reads were identified. The *ARID5B-MLL* fusion transcript has not been previously reported. No fusion events involving genes on chromosomes 2 and 14 were detected.

Validation of fusion transcripts. To evaluate the authenticity and validity of the gene fusion transcripts, Sanger sequencing of RT-PCR amplification products spanning fusion junction points was performed to validate all transcripts detected in the patient bone marrow cells. Although the RT step is able to potentially produce artifactual fusion transcripts, all three fusion transcripts, *MLL-MLLT10/AF10*, *ARID5B-MLL*, and *MLLT10/AF10-MKX*, were detected by Sanger sequencing (Fig. 2). Specifically, all fusion transcripts showing >10 junction-spanning reads were identifiable by Sanger sequencing.

Discussion

The authors herein presented a new case with inv(10)(p12.1;p12.31) and t(10;11)(q21;q23). In this case, the *MLL-MLLT10/AF10* fusion resulted from several rearrangements, including inversion followed by translocation. This patient exhibited complex rearrangements, which included inversion of 10p12.1–p12.31 followed by translocation of 11q23 to 10p12 that was associated with the generation of *MLL-MLLT10* (Fig. 3). The proximal inversion breakpoint at 10p was heterogeneous and contained a region within 10p12.1–p12.31. *MLLT10/AF10* has been reported to be involved in a complex rearrangement that resulted in an inversion of either 10p or 11q followed by translocation of 10p;11q or insertion of the inverted segment into *MLLT10/AF10* or *MLL* (7,8). *MLLT10/AF10* was also one of the 20 genes involved in complex rearrangements, with three- or four-way translocations resulting in >2 fusion alleles (8). While the 3' portions of the translocation partner genes were regularly fused to the 5'-portion of MLL, the genes involved in complex rearrangements, including *MLLT10/AF10*, were fused to the 3' portion of MLL (8). *CALM-MLLT10/AF10* and reciprocal *MLLT10/AF10-CALM* transcripts have been detected in patients with various hematological malignancies (9). Silliman *et al* (10) emphasized that truncated MLLT10/AF10 protein in normal cells and truncated MLLT10/AF10-CALM protein in leukemic cells retain a cysteine-rich motif and leukemia-associated protein (LAP) (11) and would function as dominant-negative inhibitors of full-length AF10 or associated proteins (12). There is a high likelihood that the rearrangements in the patient in the present report also involved a complex mechanism, given that the 3' portion of MKX was fused to the

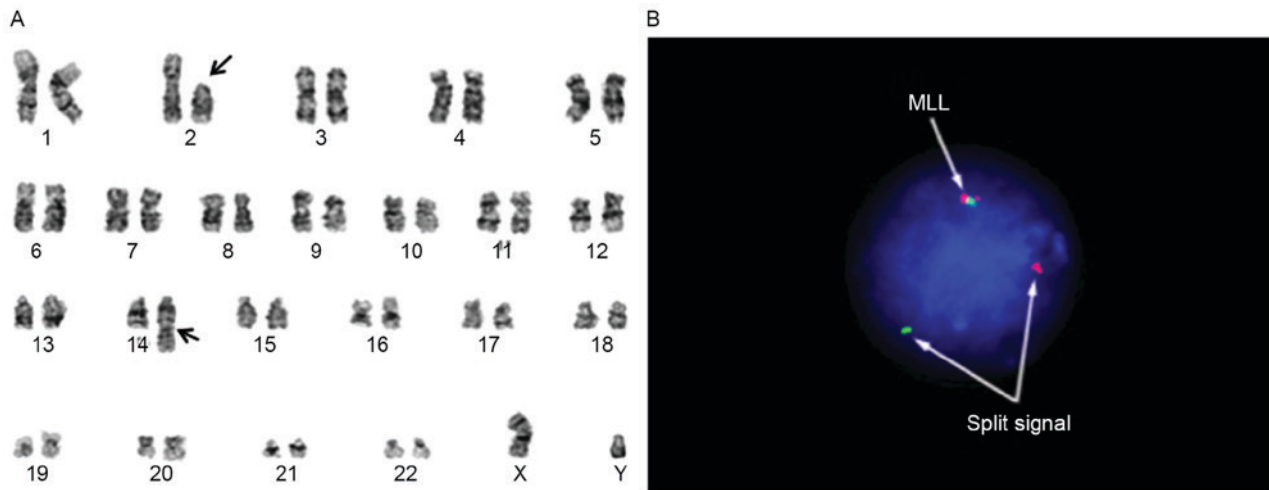


Figure 1. Cytogenetic analysis suggested the evidence for 11q23 rearrangement. (A) G-banded karyogram from bone marrow cells at diagnosis showed to be 46, XY, t(2;14)(p11.2;q32), add(11)(q23) in 14 of 20 bone marrow cells. The arrow indicates the breakpoint at 11q23. (B) Fluorescence *in situ* hybridization analysis with MLL probe (Vysis) on interphase nuclei of bone marrow cells at diagnosis. A 11q23 split-signal type was observed: One green signal and one orange signal (divided arrows). A normal signal pattern for the MLL probe (green and red fusion signals) was also observed in the bone marrow cells (arrows). MLL, mixed-lineage leukemia.

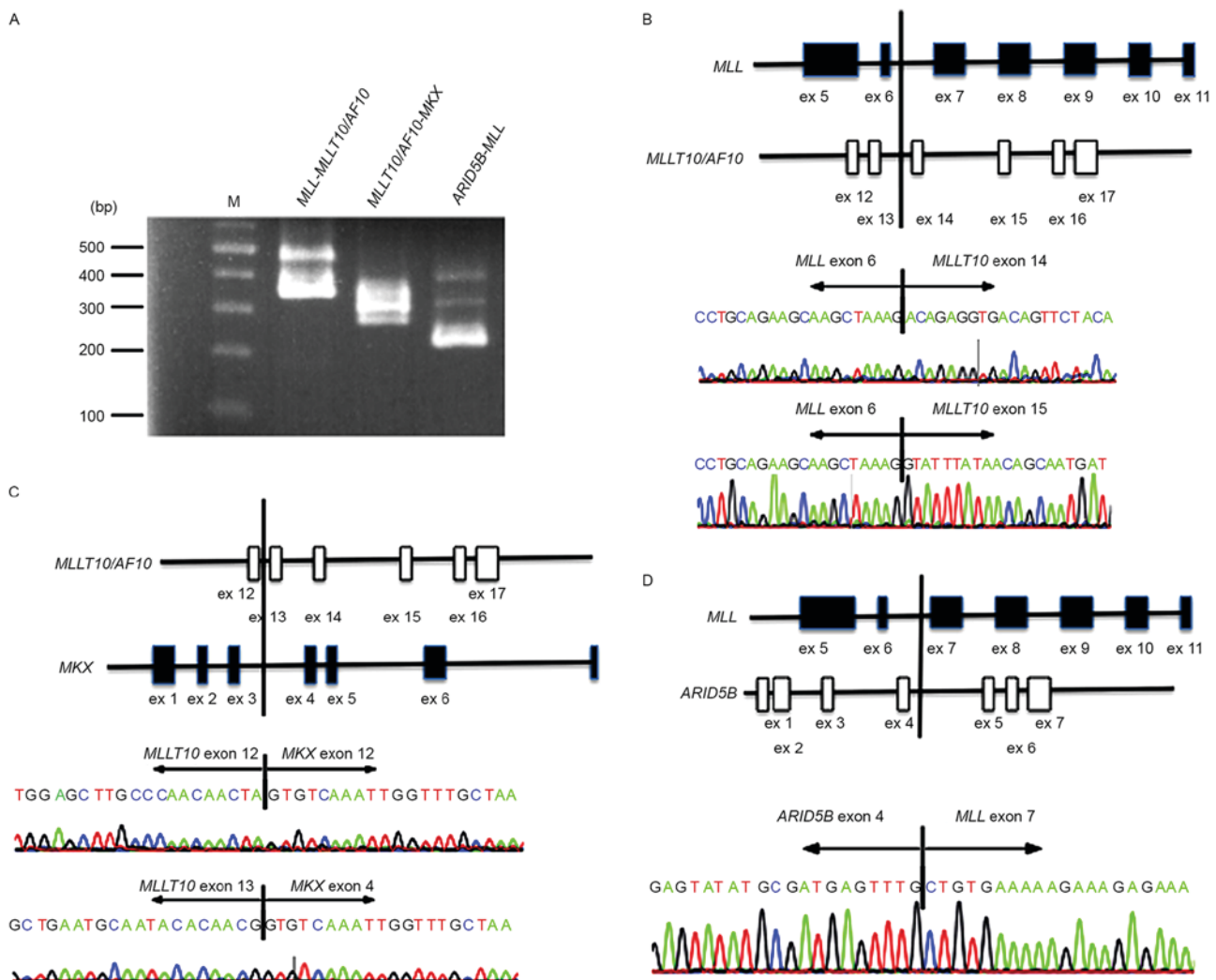


Figure 2. Validation of fusions. (A) Identification of *MLL-MLLT10/AF10*, *MLLT10/AF10-MKX* and *ARID5B-MLL* fusion transcripts in bone marrow cells from the patient using RT-PCR. Marker represents a 1 kb DNA ladder. M, size marker. (B-D) Schematic representation of reverse transcription-polymerase chain reaction products on der(10) and der(11) resulted in *MLL-M14LLT10/AF10*, *MLLT10/AF10-MKX* and *ARID5B-MLL* fusion genes. Sanger sequencing chromatograms showing the reading frame at the breakpoint and putative translation of the fusion protein in the patient's bone marrow cells. ARID5B, AT-rich interaction domain 5B; MLL, mixed-lineage leukemia; MLLT10, myeloid/lymphoid or mixed-lineage leukemia; translocated To, 10.

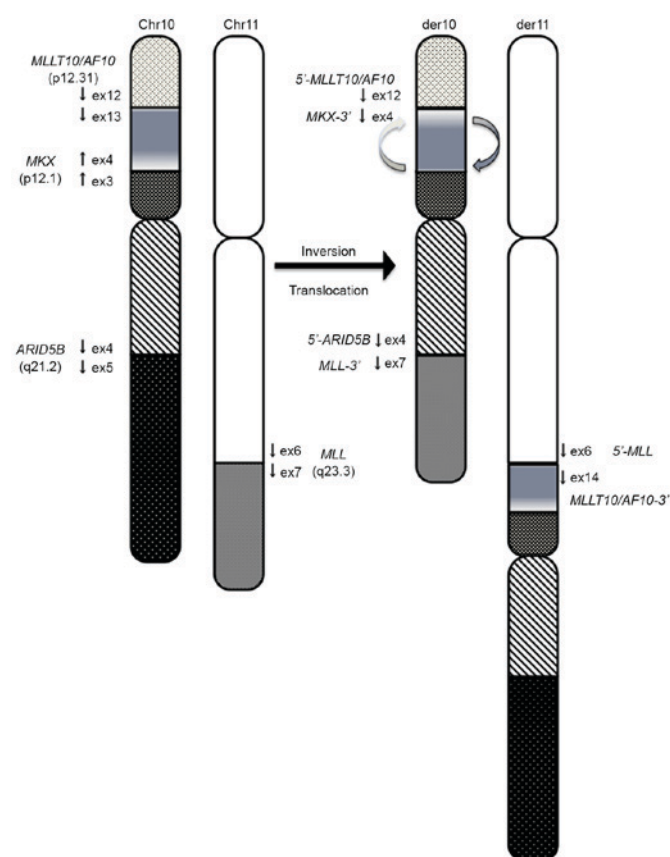


Figure 3. Proposed chromosomal mechanism, which leads to *MLL-MLLT10/AF10*, *ARID5B-MLL* and *MLLT10/AF10-MKX* rearrangements in the patient. Paracentric inversion of 10p12.31-p12.1 with a breakpoint in the *MLLT10/AF10* gene, followed by an additional break 3' of *MLL* prior to translocation of the 11q segment into *ARID5B* located at 10q21. *ARID5B*, AT-rich interaction domain 5B; *MLL*, mixed-lineage leukemia; *MLLT10*, myeloid/lymphoid or mixed-lineage leukemia; translocated To, 10.

5' portion of *MLLT10/AF10*. *MLLT10/AF10-MKX* proteins in leukemic cells retain a cysteine-rich motif and LAP. *MKX* is a member of the three-amino acid loop extension superclass of homeobox genes and is associated with cryptorchidism (13). The DNA recognition motif for *MKX* is largely dependent on a core set of amino acids in helix III, and residues at the N terminus of the homeodomain that make direct contact with the DNA (14,15). However residues at the N terminus were not maintained in the *MLLT10/AF10-MKX* fusion transcript due to the presence of an out-of frame fusion transcript, and *MKX* may be biologically inactivated. These results suggested that *MLLT10/AF10-MKX* may have a similar role in leukemogenesis as *MLLT10/AF10-CALM*.

In the present study, *ARID5B-MLL* fusion was caused by the presence of a translocation between derivative chromosomes 10q21 and 11q23. *ARID5B* gene variants were consistently shown to increase the risk of childhood ALL in various populations (16-18). The *ARID5B* gene encodes a member of the AT-rich interaction domain (ARID) family of DNA-binding proteins. The encoded protein forms a histone H3K9me2 demethylase complex together with PHD finger protein 2 to regulate the transcription of target genes involved in adipogenesis and liver development (19). Although the function of *ARID5B* in lympho-hematopoiesis has not been well studied, it may be involved in epigenetic regulation

of gene expression in hematopoietic stem cells and early lymphoid progenitors, similar to other AT-rich DNA-binding proteins (20-23). Interestingly, a single nucleotide polymorphism in *ARID5B* (RefSNP: rs10821936) was demonstrated to increase the risk of *MLL-MLLT3/AF9* (17). This finding suggests that genetic susceptibility may be associated with the differences regarding *MLL* breakpoints and partner genes. Although the role of truncated isoforms of *ARID5B* in tumorigenesis remains unknown, epigenetic dysregulation may occur in early B progenitor cells in ALL with *ARID5B-MLL* fusion. Two recent studies clearly demonstrated that reciprocal *MLL* fusion proteins may have an important role in cancer development (24,25). During follow-up analyses, a large collection of reciprocal *MLL* fusions was identified, and ~15% of these were in-frame fusions that may be readily expressed as reciprocal fusion proteins (8). All other characterized reciprocal *MLL* alleles represented out-of-frame fusions with either a chromosomal locus or a reciprocal translocation partner gene (8). However, even these events allowed the transcription and expression of a 5'-truncated *MLL* protein, termed *MLL** (26). This truncated version of *MLL* has no ability to bind Menin1, lens epithelium-derived growth factor or MYB, but it is able to carry out all enzymatic functions necessary to execute H4K16 acetylation events by associating with MOF or H3K4 methylation via the SET domain complex (27). Together with these results, the findings of the present report suggested that reciprocal *MLL* fusion proteins, including *ARID5B-MLL*, may inhibit early B-cell development and have oncogenic functions. Additional functional studies are required to understand the role of these fusion genes in the development of *MLL*-associated infantile leukemia.

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