LYMPHOID NEOPLASIA

New *MLLT10* gene recombinations in pediatric T-acute lymphoblastic leukemia

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Key Points

- In pediatric T-ALL, *MLLT10* emerged as a promiscuous gene, maintaining the critical leukemogenic OM-LZ domain in all fusions.
- *MLLT10* gene fusions were associated with a specific expression profile within the *HOXA* subgroup of pediatric T-ALL.

The *MLLT10* gene, located at 10p13, is a known partner of *MLL* and *PICALM* in specific leukemic fusions generated from recurrent 11q23 and 11q14 chromosome translocations. Deep sequencing recently identified *NAP1L1*/12q21 as another *MLLT10* partner in T-cell acute lymphoblastic leukemia (T-ALL). In pediatric T-ALL, we have identified 2 RNA processing genes, that is, *HNRNPH1*/5q35 and *DDX3X*/Xp11.3 as new *MLLT10* fusion partners. Gene expression profile signatures of the *HNRNPH1*- and *DDX3X-MLLT10* fusions placed them in the *HOXA* subgroup. Remarkably, they were highly similar only to *PICALM-MLLT10*–positive cases. The present study showed *MLLT10* promiscuity in pediatric T-ALL and identified a specific *MLLT10* signature within the *HOXA* subgroup. (*Blood*. 2013;121(25):5064-5067)

Introduction

New genomic technologies, including whole-genome analysis and gene expression profiling (GEP), dramatically improved cytogenetic-molecular classification of T-cell acute lymphoblastic leukemia (T-ALL) which affects ~15% of children with ALL.[1] At least 6 main genetic categories (ie, *TAL/LMO*, *TLX1*, *TLX3*, *NKX2-1/NKX2-2*, *MEF2C*, and *HOXA*) have been identified. The *HOXA* group includes cases with *TCRB-HOXA*, *SET-NUP214*, *MLL* translocations, and *PICALM-MLLT10*.[1,2] In a case of early T-cell precursor-ALL (ETP-ALL), the *NAP1L1*/12q21 gene, a member of the nucleosome assembly protein family, was recently identified as another *MLLT10* partner.[3]

Interestingly, PICALM-MLLT10, MLL-MLLT10, and NAP1L1-MLLT10 fusions all retained the OM-LZ domain at their C terminus.[3-5] It exerts a leukemogenic effect by interacting with chromatin modifying proteins such as the H3K79 methyltransferase hDOT1L.[6,7]

The present study focuses on 2 new *MLLT10* fusion genes in pediatric T-ALL, placing them within the *HOXA* subgroup.

Study design

A combined interphase (CI) fluorescence in situ hybridization (FISH) test (supplemental Table 1, available on the *Blood* website) was applied in 42 pediatric T-ALL patients enrolled in the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) ALL protocol. Informed consent of our work was obtained from the Bioethic Committee, University of Perugia (Prot.1. X.2011). Informed consent statements were provided according to the Declaration of Helsinki (AIEOP ALL protocol number NCT 00613457). To identify the new *MLLT10* fusions, we used a 5′-rapid amplification of cDNA ends–polymerase chain reaction (5′-RACE-PCR) (Invitrogen) and Thermoscript reverse transcription PCR (RT-PCR) system (Invitrogen) according to the manufacturer's instructions. Primers are listed in supplemental Table 2. PCR products were subcloned into pGEM-T easy vector (Promega) and sequenced with the AB3500 Genetic analyzer (Applied Biosystems). Single-nucleotide polymorphism (SNP) analysis was performed using the whole-genome cytogenetic 2.7M array (Affymetrix).

Statistical methods for microarray data (Affymetrix hgu 133 plus 2 arrays) were analyzed using the Bioconductor package for R (version 2.14.1). Data were deposited at GEO repository (series accession number GSE42765; http://www.ncbi.nlm.nih.gov/geo/). Heatmaps were created using the Ward method and Euclidean distance. The heatmap for the unsupervised analysis was created using the probe sets with the highest variances (threshold 90%), while the heatmap for the supervised analysis was created with differentially expressed probe sets. Arrays were normalized using robust multiple-array average.[8] Batch effects were removed using ComBat.[9] Differentially expressed genes were identified by the shrinkage T-statistic.[10] False-positive findings were prevented by the local false discovery rate (lFDR). Probe sets with lFDR below 0.05 were considered significant.[11,12]

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Results and discussion

CI-FISH identified *MLLT10* rearrangements in 6 of 42 (14.3%) patients. Four of the total cohort (9.5%) showed the *PICALM-MLLT10* fusion and 2 (4.8%) *MLLT10* translocations to unknown partner(s). Clinical, hematologic, cytogenetic, and molecular data of these 2 patients are shown in Table 1. The *MLLT10* FISH probe gave 3 signals in 55% of nuclei in case 1 and in 60% in case 2. In case 1, metaphase-FISH confirmed the split between the short and the long arms of 1 chromosome 10, resulting in the der(10)inv(10)(p12q25) seen at karyotype. The 10q breakpoint localized to band 25.3 in an ~12-kb region without genes (supplemental Figure 1A).

The *MLLT10* gene breakpoint was narrowed to between exons 14 and 15 in patient 1 and between exons 1 and 3 in patient 2 (supplemental Figure 1B-C). 5′ RACE-PCR and sequencing showed an *HNRNPH1-MLLT10* in-frame transcript in the first case and a *DDX3X-MLLT10* fusion transcript in the second (Figure 1A,C). These results were confirmed by RT-PCR, cloning, and sequencing. Subsequently, a diagnostic double-color double-fusion (DCDF) test was developed in both cases (Figure 1B,D). The only common additional genetic lesion was a mutated *NOTCH1* gene (Table 1).

HNRNPH1 and DDX3X are involved in RNA processing. *HNRNPH1* encodes for a member of the ubiquitous heterogeneous nuclear ribonucleoprotein subfamily (hnRNPs). It is an RNA binding protein that is involved in pre-mRNA processing, and mRNA metabolism and transport.[13] A *HNRNPH1* frameshift mutation was previously described in gastric cancer[14] and a *HNRNPH1* splice variant with protein truncation was identified in murine breast cancer cells.[15] Interestingly, a variant HNRNPH1 protein, covalently modified by O-linked acetyl hexosamine (GlcnaC), was isolated in acute leukemia with 11q23 cytogenetic changes.[16]

DDX3X is a member of the large family of RNA helicases with a DEAD box domain (Asp-Glu-Ala-Asp) that is involved in RNA transcription, splicing, mRNA transport, translation initiation, and cell-cycle regulation.[17] DEAD box RNA helicases were implicated in diverse forms of leukemia.[18,19] Recently, mutations inside and outside the DEAD box domain were detected in ~3% of patients with chronic lymphocytic leukemia.[20]

Structural analysis of *HNRNPH1-MLLT10* and *DDX3X-MLLT10* fusions showed HNRNPH1 maintained 3 RNA recognition motifs while DDX3X lost the DEAD box domain, at the N terminus. At the C terminus, MLLT10 lost 2 of 3 NLS domains in patient 1 but maintained all 3 in patient 2 (Figure 1A,C). As in other *MLLT10* fusions,[3-5] both cases retained the OM-LZ domain, which is needed to induce acute myeloid leukemia in mice bearing *PICALM-MLLT10* or *MLL-MLLT10*.[6,7] Interestingly, DOT1L inhibitors binding the OM-LZ domain were successful in controlling *MLL-MLLT10* and *PICALM-MLLT10* murine leukemias.[21]

Whether these rare *MLLT10* partners are part of a functional complex or share a common regulation pathway remains to be investigated. The Net View Tools software (http://netview.tigem.it/netview_project/netview_tools.html)[22] showed *DDX3X* was significantly coexpressed and directly linked (mutual information >0.1) to *HNRNPH1*, *PICALM*, and *NAP1L1* (supplemental Table 3).

We applied GEP to determine whether *HNRNPH1-MLLT10* and *DDX3X-MLLT10* shared leukemogenic properties with other *MLLT10* recombinations within the *HOXA* category of T-ALL.[23-24] In an unsupervised analysis of 11 T-ALL samples with the *HOXA* signature, the 6 cases with *MLLT10* rearrangements (4 *PICALM-MLLT10*, 1 *HNRNPH1-MLLT10*, and 1 *DDX3X-MLLT10*) clustered

Table 1. Clinical, immunophenotypic, hematologic, molecular, and cytogenetic data

Patient	Sex/age, y	Diagnosis	WBC, $\times 10^9$/L	*PICALM-MLLT10*	*MLL-ENL*	*MLL-AF4*	*SIL-TAL1*	*NOTCH1*	*FLT3*	Karyotype	CI-FISH	SNPs	MRD +33/+78	Chemotherapy*	Follow-up, mo
1 (UPN 1036616)	F/7	ETP-ALL	10.55	na	na	neg	neg	mut†	neg	46,XX,inv(10)(p12q25)[2] / 46,XX[17]	Split: *MLLT10*/10p12 (55%) Loss: *IKZF1*/7p12 (10%)	Normal	+/+	High risk, no transplant donor	33+
2 (UPN 1023865)	M/11	Cortical T-ALL	152.8	neg	neg	n.a.	neg	mut‡	neg	Failed	Split: *MLLT10/10p12* Loss: *PAX5,CDKN2A/B, JAK2*/9p13-24 Gain: RP11-501C14/17q21.31	Loss: 9p24.3-p11.2: from 0 to 47 508 608 bp Gain: 17q21.32-q25.3: from 45 273 751 to 78 786 769 bp	+/−	Standard risk	73+

y, years; m, months; F, female; M, male; MRD, minimal residual disease; mut, mutated; n.a., data not available; neg., negative; UPN, unique patent number; WBC, white blood cell; [ ], number of cells analyzed.
*AIEOP LLA 2000 (supplemental reference 1).
†c.4766_4767insAGCAGAACCGGAGCAGCTGCGCAACAGCTC; p.S1589_F1590insAEPEQLRNSS.
‡c.G4793C; p.R1598P; NOTCH1 mutation numbers refer to CCDS 43905.1 and NP_060087.3.

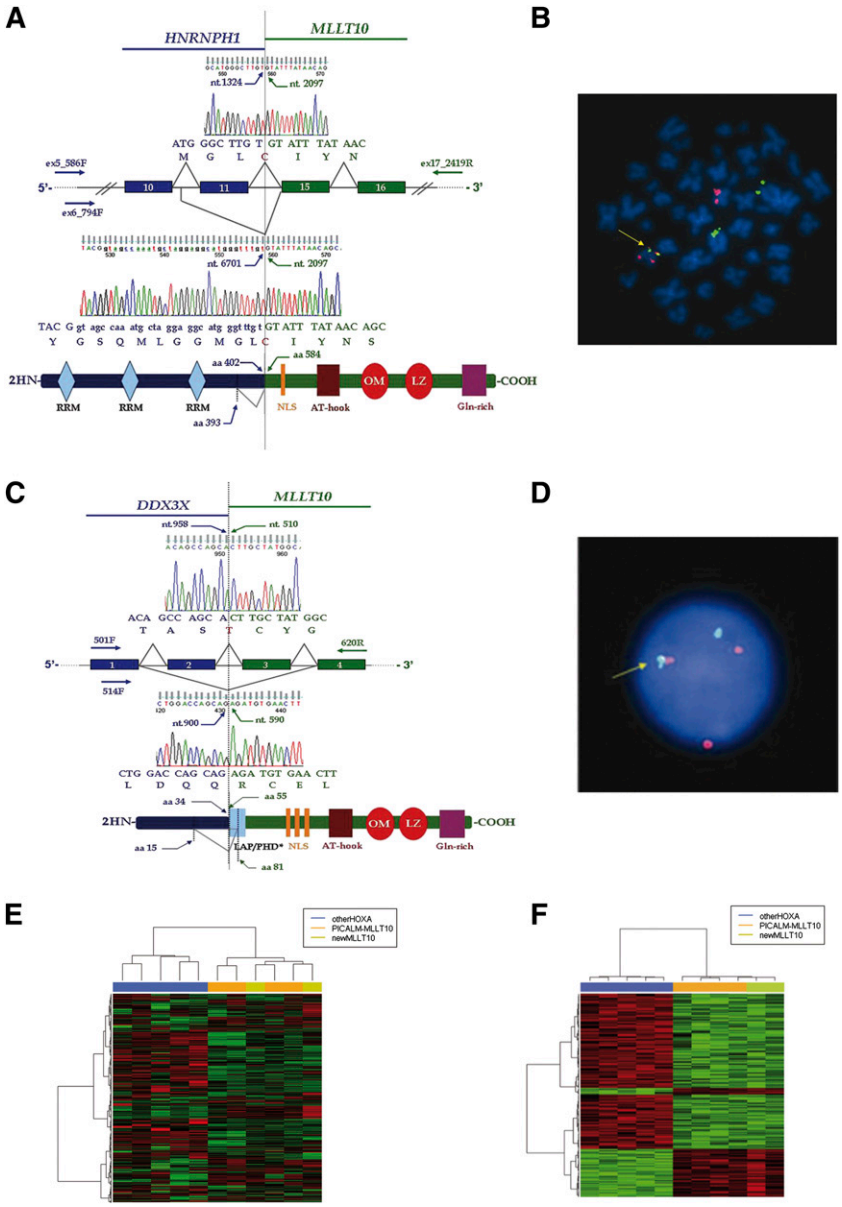

Figure 1. Molecular characterization of the two new *MLLT10* fusions. (A) Two *HNRNPH1-MLLT10* splicing isoforms were identified in patient 1. (Top) Direct sequencing showed an in-frame *HNRNPH1-MLLT10* isoform joining nucleotide 1324 (*HNRNPH1* exon 11) to nucleotide 2097 (*MLLT10* exon 15). (Bottom) Cloning and sequencing showed nucleotide 6701 (*HNRNPH1* intron 10) fused in-frame with nucleotide 2097 (*MLLT10* exon 15). Hypothetical fusion protein was shown in which HNRNPH1 maintained all 3 RRM at the N terminus and MLLT10 lost 2 of 3 NLS. MLLT10 maintained the critical OM-LZ domain at the C terminus. Primer and sequence numbers refer to GenBank accession NC_000005.9, NM_005520.2, NP_005511.1 for *HNRNPH1* and NM_004641.3, NP_004632.1 for *MLLT10*. (B) DCDF test: Probes for *MLLT10* (RP11-418C1 and RP11-249M6) in orange and for *HNRNPH1* (CTD-3223H16 and RP11-410B18) in green showed 1 fusion signal on der(10) (arrow). (C) Two *DDX3X-MLLT10* splicing isoforms were identified in patient 2. (Top) Sequencing showed an in-frame *DDX3X-MLLT10* isoform joining nucleotide 958 (*DDX3X* exon 2) to nucleotide 510 (*MLLT10* exon 3). (Bottom) Cloning and sequencing showed an in-frame isoform with nucleotide 900 (*DDX3X* exon 1) fused with nucleotide 590 (*MLLT10* exon 4). The hypothetical fusion protein lost the DDX3*X* DEAD box domain at the N terminus and maintained part of the PHD, all 3 NLS and the OM-LZ domain at the C terminus. Primer and sequence numbers refer to GenBank accession NM_001356.3, NP_001347.3 for DDX3X and NM_004641.3, NP_004632.1 for MLLT10. (D) DCDF-FISH with probes for *DDX3X* in green (RP11-1058N11, flanking 5′, and RP11-10K13, flanking 3′) and *MLLT10* in red (RP11-418C1 and RP11-249M6), showed 1 fusion signal (arrow). (E) Unsupervised analysis of 11 T-ALL *HOXA* patients. In such unsupervised analysis, patients bearing *MLLT10* rearrangements and those without *MLLT10* rearrangements (1 *MLL-ENL*, 1 *MLL-AF6*, 2 *TCRB-HOXA*, and 1 *SET-NUP214*) are naturally clustered in 2 distinct groups. *PICALM-MLLT10* patients are indicated in orange; patient 1 and patient 2 (*HNRNPH1-MLLT10*, *DDX3X-MLLT10*) in green and patients without *MLLT10* rearrangements in blue. (F) Supervised analysis was created using the significative probe sets from the comparison of *HOXA* patients with *MLLT10* rearrangements (4 with *PICALM-MLLT10* and the 2 new cases with *HNRNPH1-MLLT10*, *DDX3X-MLLT10*) vs patients without *MLLT10* rearrangements (1 *MLL-ENL*, 1 *MLL-AF6*, 2 *TCRB-HOXA*, and 1 *SET-NUP214*). Patients bearing *MLLT10* recombinations are indicated in orange or green while patients without *MLLT10* rearrangements are indicated in blue. DCDF, double-color double-fusion; LAP, Leukemia Associated Protein; NLS, nuclear localization signal; PHD, plant homeo domain; RRM, RNA recognition motif.

separately from the other 5 cases (1 *MLL-ENL*, 1 *MLL-AF6*, 2 *TCRB-HOXA*, and 1 *SET-NUP214*) (Figure 1E). *t* test analysis revealed significant (lFDR $<$ 0.05) differences in expression of 280 probe sets (supplemental Table 4). Supervised analysis with these probe sets confirmed 2 subgroups (Figure 1F). In the *HOXA* patients with *MLLT10* rearrangements, *HHEX* gene expression was higher ($>$1.5 fold-change) than in those without. *HHEX* is highly expressed in normal hematopoietic stem cells and downregulated during normal T-cell development.[25] *HHEX* overexpression was found in ETP-ALL (as seen in our case no. 1) and linked to upregulation of *MEF2C* which directly binds the *HHEX* promoter.[2] Interestingly, *HHEX* is a member of the Nirenberg and Kim–like family of class II homeobox genes. In the other subgroup of 5 patients without *MLLT10* rearrangements, gene set enrichment analysis showed enrichment of *HOXA* class I homeobox genes and target genes (supplemental Figure 2). Although these findings need to be confirmed in a larger series of T-ALL, the present results suggest that *MLLT10* recombinations underlie a specific signature, within the *HOXA* category of T-ALL.

The present study provides insights into the biological pathways involved in *MLLT10* recombinations in pediatric T-ALL. Finding 2 new *MLLT10* fusion genes, involving *HNRNPH1* and *DDX3X*, highlights the role of the *MLLT10* gene, and particularly of its OM-LZ domain in this type of leukemia.

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Authorship

Contribution: F.N. and V.P. performed karyotype analysis; V.P. selected DNA clones and performed FISH experiments; R.L.S. supervised FISH experiments and drafted the paper; L.B. designed and performed molecular experiments and drafted the paper; D.D.G. and P.G. were involved in cloning and sequencing; C.B., M.G., and G.t.K. performed GEP studies; G.C. performed SNP analysis; and C.M. was responsible for the conception of the study, supervision, and manuscript preparation.

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References

1. Van Vlierberghe P, Pieters R, Beverloo HB, Meijerink JP. Molecular-genetic insights in paediatric T-cell acute lymphoblastic leukaemia. *Br J Haematol.* 2008;143(2):153-168.
2. Homminga I, Pieters R, Langerak AW, et al. Integrated transcript and genome analyses reveal NKX2-1 and MEF2C as potential oncogenes in T cell acute lymphoblastic leukemia. *Cancer Cell.* 2011;19(4):484-497.
3. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature.* 2012;481(7380):157-163.
4. Dreyling MH, Martinez-Climent JA, Zheng M, Mao J, Rowley JD, Bohlander SK. The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. *Proc Natl Acad Sci U S A.* 1996;93(10):4804-4809.
5. Chaplin T, Ayton P, Bernard OA, et al. A novel class of zinc finger/leucine zipper genes identified from the molecular cloning of the t(10;11) translocation in acute leukemia. *Blood.* 1995; 85(6):1435-1441.
6. Okada Y, Feng Q, Lin Y, et al. hDOT1L links histone methylation to leukemogenesis. *Cell.* 2005;121(2):167-178.
7. Deshpande AJ, Rouhi A, Lin Y, et al. The clathrin-binding domain of CALM and the OM-LZ domain of AF10 are sufficient to induce acute myeloid leukemia in mice. *Leukemia.* 2011; 25(11):1718-1727.
8. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics.* 2003;19(2): 185-193.
9. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics.* 2007;8(1): 118-127.
10. Opgen-Rhein R, Strimmer K. Accurate ranking of differentially expressed genes by a distribution-free shrinkage approach. *Stat Appl Genet Mol Biol.* 2007;6:Article9.
11. Strimmer K. A unified approach to false discovery rate estimation. *BMC Bioinformatics.* 2008;9:303.
12. Strimmer K. fdrtool: a versatile R package for estimating local and tail area-based false discovery rates. *Bioinformatics.* 2008;24(12): 1461-1462.
13. Martinez-Contreras R, Cloutier P, Shkreta L, Fisette JF, Revil T, Chabot B. hnRNP proteins and splicing control. *Adv Exp Med Biol.* 2007;623: 123-147.
14. Mori Y, Sato F, Selaru FM, et al. Instabilotyping reveals unique mutational spectra in microsatellite-unstable gastric cancers. *Cancer Res.* 2002; 62(13):3641-3645.
15. Bemmo A, Dias C, Rose AA, Russo C, Siegel P, Majewski J. Exon-level transcriptome profiling in murine breast cancer reveals splicing changes specific to tumors with different metastatic abilities. *PLoS ONE.* 2010;5(8):e11981.
16. Balkhi MY, Trivedi AK, Geletu M, et al. Proteomics of acute myeloid leukaemia: cytogenetic risk groups differ specifically in their proteome, interactome and post-translational protein modifications. *Oncogene.* 2006;25(53): 7041-7058.
17. Rosner A, Rinkevich B. The DDX3 subfamily of the DEAD box helicases: divergent roles as unveiled by studying different organisms and in vitro assays. *Curr Med Chem.* 2007;14(23): 2517-2525.
18. Arai Y, Hosoda F, Kobayashi H, et al. The inv(11)(p15q22) chromosome translocation of de novo and therapy-related myeloid malignancies results in fusion of the nucleoporin gene, NUP98, with the putative RNA helicase gene, DDX10. *Blood.* 1997;89(11):3936-3944.
19. Payne EM, Bolli N, Rhodes J, et al. Ddx18 is essential for cell-cycle progression in zebrafish hematopoietic cells and is mutated in human AML. *Blood.* 2011;118(4):903-915.
20. Wang L, Lawrence MS, Wan Y, et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N Engl J Med.* 2011; 365(26):2497-2506.
21. Chen L, Deshpande AJ, Banka D, et al. Abrogation of MLL-AF10 and CALM-AF10-mediated transformation through genetic inactivation or pharmacological inhibition of the H3K79 methyltransferase Dot1l. *Leukemia.* 2013; 27(4):813-822.
22. Belcastro V, Siciliano V, Gregoretti F, et al. Transcriptional gene network inference from a massive dataset elucidates transcriptome organization and gene function. *Nucleic Acids Res.* 2011;39(20):8677-8688.
23. Dik WA, Brahim W, Braun C, et al. CALM-AF10+ T-ALL expression profiles are characterized by overexpression of HOXA and BMI1 oncogenes. *Leukemia.* 2005;19(11):1948-1957.
24. Soulier J, Clappier E, Cayuela JM, et al. HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). *Blood.* 2005;106(1):274-286.
25. Mack DL, Leibowitz DS, Cooper S, Ramsey H, Broxmeyer HE, Hromas R. Down-regulation of the myeloid homeobox protein Hex is essential for normal T-cell development. *Immunology.* 2002; 107(4):444-451.

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