High frequency of cryptic chromosomal rearrangements involving the *LMO2* gene in T-cell acute lymphoblastic leukemia

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes resulting from the transformation of T-cell progenitors. Around half of T-ALL patients harbor recurrent cytogenetic alterations, including juxtaposition of strong promoters and enhancers located in the *TCRB* (chr. 7q34) or *TCRA-TCRD* (chr. 14q11) loci with a variety of oncogenic transcription factors, such as LIM-only domain (*LMO*) genes, *LMO1* and *LMO2* resulting in their aberrant expression.[1] The LIM-only gene *LMO2* encodes a protein that participates in a transcription factor complex, which includes *E2A, GATA1*, and *LDB1*, TAL1. *LMO2* was reportedly activated in 4 cases of T-ALL arising via retroviral insertion mutagenesis in a gene therapy trial for X-linked severe combined immunodeficiency.[2-4] In addition, aberrant expression of *LMO2* has been found in 9% of pediatric T-ALL cases,[5] though higher figures have also been reported.[6] *LMO2* is activated via chromosomal translocations t(11;14)(p13;q11) or t(7;11)(q35;p13) and del(11)(p12p13) in T-ALL patients.[5] Interestingly, high *LMO2* expression levels have also been reported in many T-ALL patients without these changes, suggesting that cryptic *LMO2* rearrangements may exist in T-ALL. Recently, we identified *LMO2* rearrangements in 5 of 26 (19.2%) T-ALL cell lines including two novel cryptic non-*TCR* chromosome translocations t(3;11)(q25;p13) and t(X;11)(q25;p13), respectively activating *LMO2* by juxtaposition with *MBNL1* and *STAG2*.[7] This prompted us to investigate novel rearrangements involving *LMO2* in primary samples from T-ALL patients using fluorescence *in situ* hybridization (FISH) with tilepath BAC/fosmid clones, array-comparative genomic hybridization (CGH), and next generation sequencing (NGS) techniques.

Between July 1997 and April 2013, 409 T-ALL patients were identified following admission to JIH. A total of 264 patients' samples were included in the present study. The median age of the case series was 24 years (range 6-80 years); the majority were male (74.6%). T-cell phenotype was defined according to the EGIL criteria. Conventional R-banding was used for karyotypic analysis on bone marrow (BM) cells at diagnosis. Clonal karyotypic abnormalities were described according to ISCN.[8]

We screened all 264 T-ALL patients by FISH using BAC clones on methanol/acetic acid-fixed cells obtained from the BM cultures, as previously described.[7] For detection of *LMO2* rearrangements, dual color FISH experiments were performed with two contiguous BAC clones: RP11-646J21 and RP11-278N12, respectively labeled with Spectrum Green-dUTPand Spectrum Red-dUTP. *LMO2* rearrangements were identified in 9.1% (24 of 264) of patients. Characteristics of the 24 patients are listed in Table 1. The clinical features of *LMO2* rearranged *versus LMO2* unrearranged patients are compared in *Online Supplementary Table S1*. *LMO2* rearrangements were significantly associated with younger age, higher hemoglobin concentrations, higher lactate dehydrogenase serum levels, higher frequency of hepatomegaly or lymphadenopathy, and higher frequency of abnormal karyotype.

Among the 24 positive patients, karyotypic analysis revealed chromosomal aberrations involving 11p12-13 in 12 patients: 10 with t(11;14)(p13;q11); 2 with del(11p12). Overall, half of the *LMO2* rearrangements were cryptic by routine karyotypic analysis at a rate of 4.5% (12 of 264). *LMO2* mRNA expression levels were measured on 10 T-ALL patients with and 39 without *LMO2* rearrangements. The qRT-PCR results showed that *LMO2* transcripts were significantly higher in cases with *LMO2* rearrangements (P=0.02) than without (Figure 1A). Meanwhile, the mRNA expression levels of *LEF1, LYL1, MEF2C, STAG2, SEPT1, TLX1*, and *TLX3* were also measured by qRT-PCR in these patients, showing no differences between patients with and without *LMO2* rearrangements, with the exception of LEF1. Our findings show that patients with *LMO2* rearrangements had higher *LEF1* transcripts (P=0.015) (Figure 1A), which raises the possibility that *LMO2* interactome includes *LEF1* in T-ALL, as reported in B-cell lymphomas.[9]

Furthermore, to explore undetectable cytogenetic abnormalities, we performed integrative genomic and transcriptional analyses on these 24 T-ALL patients with *LMO2* rearrangements. FISH with RP11-646J21/278N12 probes revealed del(11p13p13) in 6 patients including 2 with del(11p12) according to routine karyotyping (Table 1). Array-CGH analysis confirmed respective 95 Kbp and 475 Kbp deletions including the upstream *LMO2* region at 11p13 in 2 T-ALL samples (Figure 1B).

In addition, we performed whole genome sequencing in 2 *LMO2* rearranged T-ALL patients (cases 6 and 10) without t(11;14)(p13;q11) or del(11)(p12p13). We performed sequencing to a mean coverage of 50x in each sample. In case 6 (normal karyotype), we identified fusion between 11p13 (33,856,828 bp) and 7q34 (142,494,025 bp). Two segments, respectively 7-bp and 16-bp, of unknown origin were inserted at 11p13 and 7q34 breakpoints. PCR and bi-directional Sanger sequencing confirmed the presence of chimeric product. The 11p13 breakpoint lay ~23 Kbp downstream of *LMO2*, while the 7q34 breakpoint lay within the *TCRB* gene. Dual color FISH experiments confirmed a balanced translocation between 7q34 and 11p13 (Figure 1C). Thus, we identified a rather rare translocation, t(7;11)(q35;p13), in case 6, which has been reported only in a very few T-ALL patients hitherto.[10]

In case 10, we identified a fusion between 11p13 (33,957,035 bp) and 14q32 (98,842,615 bp). PCR and bi-directional Sanger sequencing confirmed the presence of chimeric product. The 11p13 breakpoint lay ~43 Kbp upstream of *LMO2*. The 14q32 breakpoint was located between C14orf64 and C14orf177 genes, ~793 kbp downstream of *BCL11B*. We recently described a cluster of powerful T-cell enhancers in 3´-*BCL11B* which can activate homeobox oncogenes *NKX2-5* and *HOX11L2* by juxtaposition in cytogenetically identical t(5;14)(q35;q32.2).[11-13] The corresponding region in mice has been shown to control specificity of T-cell expression therein.[14] It is interesting to note that this patient had the highest *LMO2* transcription level as shown by qRT-PCR in the 49 T-ALL patients mentioned above. We, therefore, propose that the *LMO2* gene is deregulated by juxtaposition with 3´-*BCL11B* via a novel t(11;14)(p13;q32.2) rearrangement (Figure 1D). In T-ALL, cytogenetic alterations juxtaposing *LMO2* with strong promoters and enhancers of T-cell receptor loci are recognized as the main activating mechanism. Placement of patient breakpoints often provides clues to the underlying leukemogenic mechanisms involved. The respective breakpoint regions at 11p13/*LMO2* and 14q32.2/*BCL11B* are shown in Figure 1E. While the breakpoint at 14q32 lay amid the far distal downstream cluster which we reported previously where NK-family homeobox genes are activated,[13] that at 11p13 lay upstream of those involved in *TCR*-

LMO2 rearrangements where it clustered together with *MBNL1* and *STAG2* additional non- *TCR-LMO2* partners which we described recently.[7] FISH analysis with BAC clones in 11p13 and whole chromosome painting confirmed the translocation between *LMO2* with the short arm of chromosome 2 in another patient with 47,XY,t(1;1)(p33;q41),t(2;11)(p15;p13),i(7q),+12[10] (case 15) (Figure 1F). Chromosome 2p15 has yet to be assigned a recurrent oncogene target in T-ALL to serve as candidate *LMO2* partner in this case. Taken together, these findings imply that the imputed activation of *LMO2* by non-*TCR* loci is mechanistically distinct from canonical

Table 1. Clinical and biological characteristics of T-ALL patients.

	The entire group	*LMO2*(+)	*LMO2*(-)	*P*
Total (n)	264	24	240	
Clinical features				
Sex (n)				0.128§
Male	197	21	176	
Female	67	3	64	
Age at diagnosis (years)				0.002*
Median	24	17.5	25	
Range	6-80	7-60	6-80	
WBC(10×10^9/L)				0.135*
Median	36.7	51.9	28.55	
Range	0.7-899.38	2.6-593	0.7-899.38	
Hb(g/L)				0.001*
Median	99	123	98	
Range	36-195	69-164	6.2-195	
PLT(10×10^9/L)				0.672*
Median	60	47	62	
Range	4-390	8-196	4-390	
LDH(U/L)				0.027*
Median	508	2425	432.5	
Range	115-9976	180-6049	115-9976	
BM blasts (%)				0.821*
Median	80.5	82	80.5	
Range	20-98	20-98	20-98	
Splenomegaly, %	52.7	76.9	48.8	0.054§
Hepatomegaly, %	19.3	58.3	12.7	0.001§
Lymphadenopathy, %	57.3	92.3	51.3	0.005§
FAB, n.				0.544§
L1	107	10	96	
L2	95	9	92	
NA	62	5	52	
Cytogenetics, n (%)				0.001§
Normal karyotype	152 (57.57%)	6 (25%)	146 (60.83%)	
Abnormal karyotype	102 (38.63%)	17 (70.83%)	85 (35.42%)	
Unsuccessful karyotype	10 (3.78%)	1 (4.17%)	9 (3.75%)	
Mutations				
FBXW7 mutation (n=88)				0.168§
FBXW7 (+)	8	3	5	
FBXW7 (-)	80	10	70	
IL7R mutation (n=88)				0.252§
IL7R (+)	4	0	4	
IL7R (-)	84	13	71	
NOTCH1 mutation (n=88)				0.956§
NOTCH1 (+)	40	6	34	
NOTCH1 (-)	48	7	41	
PHF6 mutation (n=88)				0.265§
PHF6 (+)	12	0	12	
PHF6 (-)	76	13	63	
WT1 mutation (n=88)				0.252§
WT1 (+)	4	0	4	
WT1 (-)	84	13	71	
Expression				
LMO2 expression (n=49)	49	10	39	0.02§
LEF1 expression (n=49)	49	10	39	0.015§

*WBC: white blood cell; Hb: hemoglobin; PLT: platelets; LDH: lactate dehydrogenase; BM: bone marrow. P: *rank sum test; §χ^2 test.*

translocation disease, a conclusion of potential therapeutic relevance.

Interestingly, we identified simultaneous involvement of *TCRB* and *TCRA-TCRD* in case 17 by FISH screening. Further FISH characterization indicated that *LMO2* is activated via formation of t(7;11)(q35;p13), and *MYC* via t(8;14)(q24;q11) in this patient (Figure 1G).

To determine the association of *LMO2* rearrangements with other recurrent gene mutations in T-ALL, we investigated gene mutations by PCR and direct Sanger sequencing in a cohort of 88 T-ALL patients for whom genomic DNA was available, including 13 with *LMO2* rearrangements. After excluding known polymorphisms and silent mutations, mutations of *FBXW7, IL7R, NOTCH1, PHF6* and *WT1* were respectively detected in 8 (9.1%), 4 (4.5%), 40 (45.5%), 12 (13.7%), and 4 (4.5%) of these 88 patients (*Online Supplementary Table S1*). There were no significant differences in the incidence of *FBXW7* (P=0.168), *IL7R*

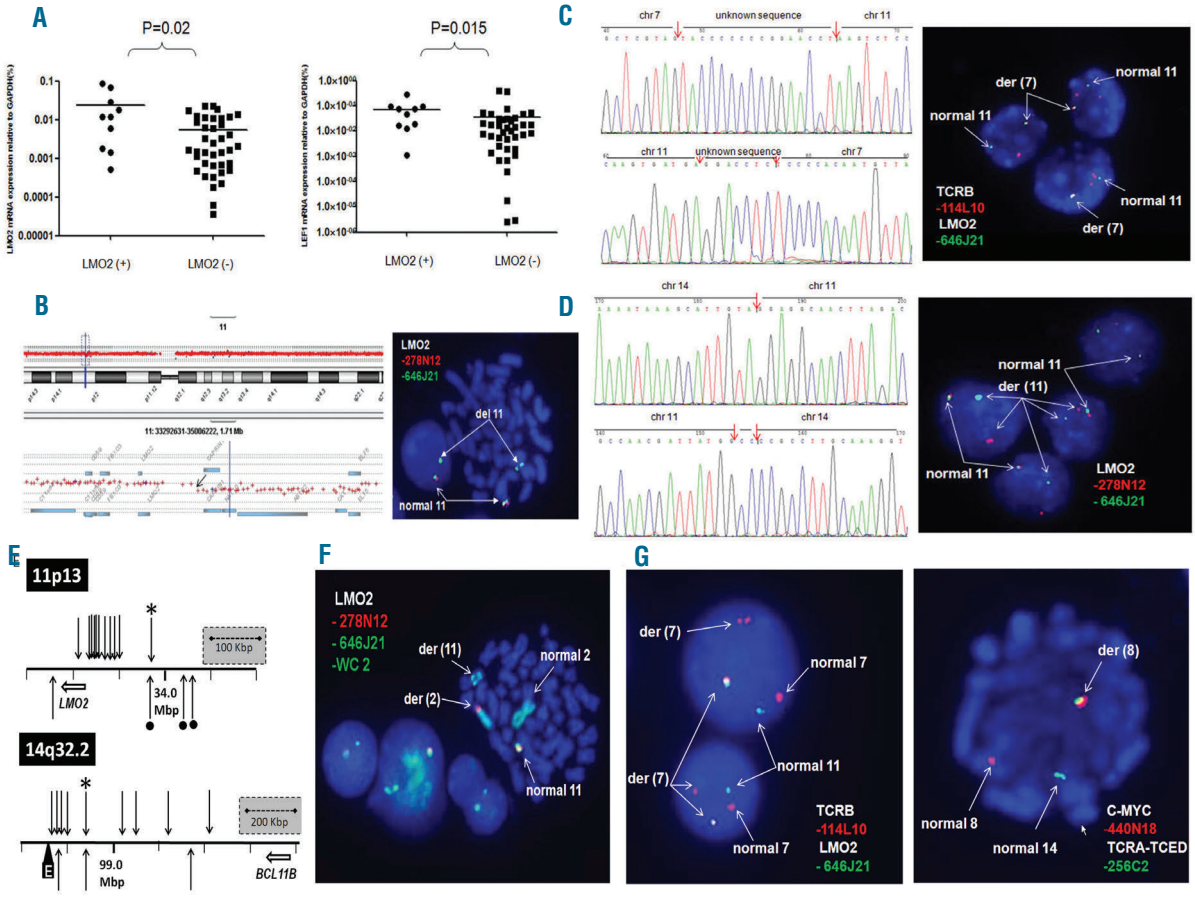

Figure 1. Integrative genomic and transcriptional analyses of *LMO2* rearrangements in T-ALL patients. (A) The q(uantitative)R(everse)Transcription-PCR results (left) showed that *LMO2* transcripts were significantly higher in cases with *LMO2* rearrangements (P=0.02) than without. Meanwhile, *LEF1* transcripts (right) were significantly higher in cases with *LMO2* rearrangements (P=0.015) than without. (B) FISH with RP11-646J21 (green) and RP11-278N12 (red) probes and array-CGH analysis revealed a 475 Kbp of del(11p13p13) including the upstream *LMO2* region in T-ALL samples. (C) Whole genome sequencing (WGS) was performed using the Illumina Hiseq 2500 system (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. We identified a fusion between 11p13 (33,856,828 bp) with 7q34 (142,494,025 bp) in case 6 with normal karyotype (left). Dual color FISH experiments with probes RP11-114L10 (*TCRB*, red) and RP11-646J21 (*LMO2*, green) confirmed the balanced translocation between 7q34 and 11p13 (right). (D) WGS identified a fusion between 11p13 (33,957,035 bp) and 14q32 (98,842,615 bp) in case 10 with complex karyotype (left). Dual color FISH experiments with RP11-646J21 (green) and RP11-278N12 (red) probes confirmed the involvement of *LMO2* in this patient (right). (E) *LMO2* and *BCL11B* breakpoints in T-ALL. Diagram shows distribution of translocation breakpoints at chromosome 11p13 and 14q32.2 previously reported in T-ALL.[7,13] Arrows indicate patient breakpoints above and cell lines below co-ordinate plots. The t(7;11)(q35;q13) and t(11;14)(q13;q32) breakpoints mapped in this report are indicated by a diamond and asterisk, respectively. The black wedge ("E") shows a remote downstream enhancer region characterized by us previously,[13] which coincides with the distal *BCL11B* breakpoint cluster region boundary. Note placement (right figure) of the *LMO2-BCL11B* breakpoint amid other *BCL11B* partners, TLX3 and NKX2-5, consistent with analogous activation mechanisms for all three oncogene targets. Note also contrasting non-canonical placement (left figure) of the *LMO2-BCL11B* patient breakpoint upstream of *LMO2* breakpoints all of which involved *TCR* loci. The same patient breakpoint lay instead amid other non-*TCR* cell-line breakpoints (bullets), all located more distally upstream of *LMO2*, implying mechanistic differences between the oncogene activation mechanisms of *TCR* and non-*TCR LMO2* translocations. (F) FISH with RP11-646J21 (green) and RP11-278N12 (red) probes and whole chromosome painting probe for chromosome 2 revealed a translocation between *LMO2* with the short arm of chromosome 2 in case 15 with 47,XY,t(1;1)(p33;q41),t(2;11)(p15;p15),i(7q),+12[10]. (G) FISH analysis revealed simultaneous involvement of *TCRB* and *TCRA-TCRD* in case 17. Dual color FISH experiments with probes RP11-114L10 (*TCRB*, red) and RP11-646J21 (*LMO2*, green) confirmed the rearrangement between 7q34 and 11p13 (left). Meanwhile, FISH with probes RP11-440N18 (*MYC*, red) and RP11-256C2 (*TCRA-D*, green) confirmed the rearrangement between 8q24 and 14q11 (right).

(*P*=0.252), *NOTCH1* (*P*=0.956), *PHF6* (*P*=0.265), and *WT1* mutated cases (*P*=0.252) with and without *LMO2* rearrangements. We also sequenced the entire coding region of *LMO2* in 117 T-ALL patients and found no somatic mutation. Taken together, *LMO2* rearrangements were identified in 9.1% (24 of 264) of T-ALL patients of which 50% (12 of 24) were deemed cryptic. The *LMO2* transcripts were significantly higher in cases with *LMO2* rearrangements than without. Moreover, we detected non-*TCR* chromosome translocations activating *LMO2* in 2 T-ALL patients, suggesting that non-*TCR* chromosome translocations activating *LMO2* are recurrent in T-ALL at significant levels. It is worthy of note that we identified a novel t(11;14)(p13;q32.2) translocation which activates *LMO2* by juxtaposition with remote leukemic enhancers of 3'-*BCL11B* using whole genome sequencing. Our results indicate that *LMO2* is a novel partner gene of *BCL11B* in T-ALL besides *TLX3* and *NKX2-5*.

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