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**HOX11L2/TLX3 is transcriptionally activated through T-cell regulatory elements downstream of BCL11B as a result of the t(5;14)(q35;q32)**

Xin-Ying Su, Véronique Della-Valle, Isabelle Andre-Schmutz, Claudie Lemercier, Isabelle Radford-Weiss, Paola Ballerini, Michel Lessard, Marina Lafage-Pochitaloff, Francine Mugneret, Roland Berger, Serge P. Romana, Olivier A. Bernard, and Virginie Penard-Lacronique

From the Institut National de la Santé et de la Recherche Médicale (INSERM), E0210, Paris, France; University René Descartes, Paris, France; INSERM, U768, Paris, France; INSERM, U548, Grenoble, France; CEA-DRDC-laboratoire "Immunochimie," Grenoble, France; Assistance Publique-Hôpitaux de Paris (AH-HP), Laboratoire de Cytogénétique, Hop Necker-Enfants Malades, Paris, France; AP-HP, Service d’Hematologie Biologique, Hop Trousseau, Paris, France; Laboratoire d’Hématologie, Hop de Hautepierre, Strasbourg, France; Laboratoire de Cytogénétique, Institut Paoli-Calmettes, Marseille, France; Université de la Méditerranée, Marseille, France; and Laboratoire de cytogénétique, CHU de Dijon, Dijon, France.

**Abstract**

The t(5;14)(q35;q32) chromosomal translocation is specifically observed in up to 20% of childhood T-cell acute lymphoblastic leukemia (T-ALL). It affects the BCL11B/CTIP2 locus on chromosome 14 and the RANBP17-TLX3/HOX11L2 region on chromosome 5. It leads to ectopic activation of TLX3/HOX11L2. To investigate the reasons of the association between t(5;14) and T-ALL, we isolated the translocation breakpoints in 8 t(5,14) patients. Sequence analyses did not involve recombinase activity in the genesis of the translocation. We used DNAseq1 hypersensitive experiments to locate transcriptional regulatory elements downstream of BCL11B. By transient transfection experiments, 2 of the 6 regions demonstrated cis-activation properties in T cells and were also effective on the TLX3 promoter. Our data indicate that the basis of the specific association between t(5;14) and T-ALL lies on the juxtaposition of TLX3 to long-range cis-activating regions active during T-cell differentiation.

**Introduction**

Maturation of immunoglobulin (Ig) and T-cell receptor (TCR) genes is associated with somatic V(D)J recombinase rearrangements occurring during lymphoid development. A protein complex containing both ubiquitously expressed and lymphoid-specific proteins (RAG1, RAG2, and TdT) catalyzes this process. The complex interacts with recombination sequences (RSs) that limit the segments to be rearranged. During the recombination process, the extremities are nibbled, and additional nucleotides (Ns) are added without template by the TdT. During this process, which includes double-strand DNA break, the Ig and TCR genes are prone to illegitimate rearrangements and chromosomal translocations observed in human T-cell acute lymphoblastic leukemia (T-ALL) often involve the site of the TCR genes. Molecular analyses of the chromosomal breakpoints of these translocations often showed...
hallmark of recombinase activity and allowed the characterization of numerous T-ALL oncogenes.

Many of the oncogenic events specific for T-cell leukemogenesis result in the activation of transcription factors, such as HOX11/TLX1, LMO2, or TAL1/SCL. A frequent event in T-ALL is the occurrence of a 90-kb deletion of chromosome 1 sequences, juxtaposing the first exon of the SIL gene, to the 5' part of the TAL1 gene. This rearrangement is observed in up to 20% of childhood T-ALL samples, and not in other ALL subtypes. The fusion points of the deletion bear the hallmarks of recombinase activity, which is therefore thought to be responsible for this rearrangement and provides the grounds for its specific association with T-ALL. Illegitimate recombinase activity is also responsible for at least some of the deletion affecting the P16-ARF tumor-suppressor locus observed in a T-ALL.

Ectopic expression of the homeobox transcription factor TLX3 was recently reported in childhood T-ALL, virtually always in association with genomic rearrangement of its locus. It is mainly associated with a cryptic t(5;14)(q35;q32) chromosomal translocation that recombines the RANBP17-TLX3 and BCL11B loci. To uncover the mechanisms responsible for the translocation and ectopic expression of TLX3, and the reason for the specific association between t(5;14) and T-ALL, we analyzed the nucleotide sequence at the breakpoint of 8 examples of t(5;14). In addition, we identified cis-transcriptional regulatory elements lying downstream of BCL11B, which could be involved in the up-regulation of TLX3 following the translocation.

Materials and Methods

Samples were obtained with informed consent, in accordance with the Declaration of Helsinki. Approval for this study was given by the INSERM review board. The hematologic and immunophenotypic characteristics and conventional karyotypes of the 8 T-ALL patients with t(5;14)(q35;q32) are listed in Table S1, available on the Blood website (see the Supplemental Materials link at the top of the online article). The procedure for DNAse1 experiments is described in Figure S2. DNA analyses were performed using standard techniques. DND41, HPB-ALL, and Jurkat are T-ALL–derived cell lines; DND41 and HPB-ALL bear variant t(5;14). The primers used are listed in Table S2. Inverse polymerase chain reaction (PCR) was performed as described previously, and classical PCR was then used to confirm breakpoint location and sequence. Sequence analyses were performed locally or at http://genome.ucsc.edu or http://www.ncbi.nlm.nih.gov/gene. Appropriate fragments were cloned in pGL3 backbone (Promega, Madison, WI). Cells (2.5 x 10^5) were transfected with 0.5 to 2 μg reporter gene constructs using Lipofectine (Invitrogen, Frederick, MD), and the luciferase activity was determined using luciferase assay system (Promega) after 24 hours. A representative experiment is shown, but every experiment was repeated at least 3 times in duplicate. The results were similar with or without normalization (using a thymidine kinase promoter–driven -galactosidase reporter).

Results and Discussion

Cloning of the chromosomal translocation breakpoints

Approximate mapping of the breakpoints was done by fluorescence in situ hybridization (FISH), analyses and more precise localization was achieved by Southern blotting analysis using single-copy probes (data not shown and Figure 1A). The translocation breakpoints were amplified from patient's genomic DNA using an inverse PCR approach and
analyzed by direct sequencing. Both translocation breakpoints (der5 and der14) could be analyzed in 7 cases and the fusion points exhibited complex features in only patient 1 (Figure S1). Most of the fusion points exhibited N nucleotides. In patients 3 and 7, no N nucleotides were observed, suggesting that the translocation could have occurred in a cell that did not express the TdT. Of importance, no convincing RS-like sequences were observed at the translocation breakpoints, suggesting that the recombinase activity is not involved in the occurrence of the translocation.

**DNAse1 hypersensitive site (HSS) mapping**

It has been proposed that the transcriptional activation of TLX3 could result from cis-activation of the gene by a BCL11B transcriptional regulatory element, juxtaposed to TLX3 following the translocation.\(^ {12-15}\) Within the hematopoietic system, expression of the BCL11B gene is restricted to the T-cell differentiation pathway.\(^ {5,16}\) It encodes a transcription factor essential to thymocyte differentiation.\(^ {17}\) The location of the translocation breakpoints in patients 1 and 6 pinpointed to a 120-kb region of chromosome 14 susceptible to contain this putative minimal region. A previously described breakpoint\(^ {15}\) allowed us to narrow down this region to 58kb lying more than 920-kb downstream of BCL11B polyadenylation site. We used DNAse1 hypersensitive experiments to identify active regulatory elements in this region. As shown in Figure 1B, 5 HSSs were observed in T-ALL cell lines (DND41 and Jurkat), but were absent or weaker in the non–T-cell lines Nalm6 and K562 (Figure S2).

To investigate for cis-activating properties, 1.5-kb fragments encompassing the HSSs were subcloned in pGL3-luciferase vector and tested for activation of the SV40 promoter. Only 2 regions (H3 and H4) showed enhancement of SV40 promoter activity in HPB-ALL (Figure 2A). Fine mapping of the HSS and luciferase reporter assays allowed us to map the active sequences within 464-bp (H3*) and 482-bp (H4*) fragments (data not shown and Figure 2B). As expected, cis-activation was observed in T-cell lines, but not in B-cell or myeloid cell lines. We next wanted to ensure the activity of these sequences on the TLX3 promoter. A 504-bp fragment within the 5' side of TLX3 was subcloned in promoter-less pGL3-luciferase vector (Figures S3 and S4). As shown in Figure 2C, H3* and H4* sequences stimulated the TLX3 promoter, which exhibited only a weak activity on its own in T-cell lines. Taken as a whole, our results identify T-lymphoid-restricted (within the hematopoietic system) cis-activating elements downstream of BCL11B that are juxtaposed to the TLX3 locus as a result of the t(5;14).

Our observations suggest that the recombinase activity is not responsible for the occurrence of the t(5;14) in contrast with the SIL-TAL1 deletion. They rather suggest that the reason for the association between t(5;14) and T-ALL is the juxtaposition of cis-transcriptional regulatory elements that are specifically active during T-cell differentiation. In keeping, murine Bcl11b is very highly expressed at starting from DN2 (and latter) stage of thymic development (data not shown).

The t(5;14) situation is the most frequent example observed in T-ALL that results in bona fide cis-activation due to enhancer juxtaposition. The frequent SIL-TAL1 fusion leads to transcription of TAL1 from the SIL promoter.\(^ {2}\) The breakpoints of chromosomal translocation affecting the TCR locus frequently affect the 5' part of the oncogene on the partner chromosome, and result in the transcription from diversity region or cryptic promoters close to the translocation breakpoints.\(^ {18-21}\) We provide here only qualitative notions, and additional work will be necessary to fully characterize the BCL11B gene cis-regulating elements.\(^ {22,23}\)
However, it is tempting to speculate that the activity of these transcriptional regulatory regions represents a therapeutic target in t(5;14) patients.

Authorship

Contribution: X.-Y.S., V.D.-V., I.A.-S., C.L., and I.R.-W. performed research and analyzed the data; P.B., M.L., M.L.-P., and F.M. provided vital reagents; R.B. and S.P.R. analyzed the data and reviewed the paper; O.A.B. and V.P.-L. designed research, analyzed data, and wrote the paper.

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Dr Su's current address is the Shanghai Institute of Hematology, Rui Jin Hospital, Shanghai, People’s Republic of China.

Footnotes

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Correspondence: Virginie Penard-Lacronique, INSERM E0210, IRNEM; Hôpital Necker-Enfants Malades, 149 rue de Sèvres, 75015, Paris, France; e-mail: penard@necker.fr

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Figure 1. Structural analyses of the loci involved in t(5;14) identify putative transcriptional cis-regulatory elements. (A) Localization of the translocation breakpoints on chromosome 5 (top) and on chromosome 14 (bottom). Vertical arrows indicate the location of the breakpoints. The number refers to the patient's number. The majority of chromosome 5 breakpoints is located in a 69-kb area between exons 20 to 25 (shown by vertical lines) of RANBP17. A horizontal arrow indicates the sense of transcription of the genes. The chromosome 14 breakpoints are spread over 842 kb downstream of BCL11B, in a region devoid of any obvious gene. The nucleotide sequences of the breakpoints are presented in Figure S1. Note that the breakpoint of patient 1 is located in the 3' untranslated region of TLX3. The chromosome 14 region that is juxtaposed in cis to the TLX3 gene in every t(5;14) patient characterized here is located between the breakpoints of patients 1 and 6. F1 indicates the breakpoint of a complex t(7;14;15) reported previously that allows the breakpoint to narrow the "critical" region to a 58-kb area indicated by a black rectangle (B) DNase1 mapping experiments in the chromosome-14 region predicted to contain transcriptional regulatory elements. (Top) Map of the 58-kb chromosome-14 area analyzed in this experiment, showing EcoRV (EV) and BamHI (BHI) sites and the probes used. The numbers refer to chromosome 14 genomic sequences (NCBI Build36; NCBI, Bethesda, MD). (Bottom) Southern blot analysis of DNase1 hypersensitivity experiments using DND41 cells. Increasing concentrations of DNase1 reveal 5 hypersensitive sites (the corresponding fragments are shown by arrows). The sizes of the germ-line fragments are indicated. Additional experiments are presented in Figure S2.
Figure 2. Identification of cis-regulator elements active on the TLX promoter active in T-cell lines. (A) Transcriptional regulatory activity of HSSs 1 to 5. Fragments encompassing the HSSs were subcloned in pGL3 (S-pGL3: containing an SV40 promoter) and tested by transient transfection in HPB-ALL. Luciferase activities are in arbitrary units (AU) and not normalized. (B) Fragments from H3 and H4 were tested for transcriptional activity using S-pGL3 luciferase reporter vector in HPB-ALL (T-ALL; left panel) and Nalmé (BCP-ALL; right panel) cell lines. Three different concentrations of vector were used. Values are indicated as fold of activation with respect to 0.5 µg S-pGL3 vector independently in each cell line. Data were normalized using an β-galactosidase reporter. (C) H3* and H4* are active on SV40 (left panel) and TLX3 (right panel) promoters. Normalized values are indicated as fold of activation with respect to 0.5 µg S-pGL3 vector (left panel) or 0.5 µg T-pGL3 (right panel). The nucleotide sequence of fragments H3* and H4* is shown in Figure S5. Error bars indicate standard deviations.