Transcriptional activation of the cardiac homeobox gene *CSX1/NKX2-5* in a B-cell chronic lymphoproliferative disorder

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ABSTRACT

Homeobox containing transcription factors are frequently deregulated in human hematologic malignant diseases either indirectly through an abnormality of an upstream factor, or directly through rearrangement of the gene itself. Study of T-cell acute lymphoblastic leukemia identified the related non-clustered homeobox transcription factors, TLX1 and TLX3, as frequently ectopically expressed as a result of chromosomal translocations. We report the deregulation of a non-clustered homeobox gene in a new type of t(5;14)(q35;q11) translocation in a mature peripheral B-cell leukemia. This translocation results in the ectopic expression of the *CSX1/NKX2-5* gene on chromosome 5q35 due to its juxtaposition to the TCR δ gene on chromosome 14q11. Expression of the CSX1/NKX2-5 protein conferred enhanced replating potential to transduced murine bone marrow cells. Our study establishes that deregulation of homeobox encoding genes is not restricted to acute leukemic proliferations, but is also observed in chronic malignant diseases.

Key words: chromosomal translocation, homeobox, transcriptional activation, chronic lymphocytic leukemia.

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Introduction

Chromosomal abnormalities observed in human malignant diseases are a frequent mechanism of gene deregulation or disruption. Chromosomal translocations involving the T-cell receptor (*TCR*) and immunoglobulin (*BCR*) genes are exclusively seen in lymphoid tumors.[1] Generally TCR gene translocations are closely associated with T-cell lymphoid tumors, whereas BCR translocations are associated with B-cell malignant diseases. The studies of chromosomal translocations allowed the identification of several human oncogenes that can also be targeted by other types of oncogenic events, like mutations. An example is the *NOTCH1* gene, isolated in humans because of its rearrangement with the TCR β chain gene in the rare t(7;9)(q34;q34) translocation and now known to be altered in more than 50% of T-cell acute lymphoblastic leukemia (ALL).[2]

One of the major targets in leukemogenesis is the family of homeobox-containing genes which encode DNA-binding proteins. They can be deregulated by various mechanisms, directly or indirectly. Transcriptional deregulation of clustered homeobox genes is observed as an indirect result of chromosomal translocations involving the *MLL* gene, a regulator of the Trithorax family, or from yet uncharacterized causes in various leukemic subtypes.[3] *PBX1*, an homeobox-cofactor of HOX gene, is fused to the E2A gene by the t(1;19)(q23;p13) translocation of B-cell ALL.[4]

The *HOX11/TLX1* orphan gene family is inappropriately expressed in about 30% of T-ALL samples.[5-7] TLX1, located on chromosome 10q24, is ectopically expressed upon its rearrangement with the TCR δ, or TCR β, chain genes in 10% of T-ALLs. The related *HOX11L2/TLX3* gene, located on chromosome 5q35, is rearranged with the TCR δ gene on chromosome 14 (band q11) in only a few cases of ALL.[8] However, it is targeted by the cryptic t(5;14)(q35;q32) translocation, which affects the CTIP2/BCL11B locus on chromosome 14 (band

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q32) in more than 20% of childhood T-ALLs.[9,10] Finally, a new chromosome 5q35 breakpoint has been reported close to the *CSX1/NKX2-5* homeobox gene, approximately 2 megabases telomeric to *TLX3*, which juxtaposes *NKX2-5* to *BCL11B* locus derived sequences in two cell lines[11] and to *TCRD* in a T-ALL patient.[12]

In a search for additional structural abnormalities of the long arm of chromosome 5, we identified a t(5;14)(q35;q11) translocation in a B-cell chronic lymphoproliferative disorder. We now report the molecular characterization of this translocation and demonstrate the involvement of the *TCR* δ gene on chromosome 14 and the *NKX2-5* gene on chromosome 5.

Case report

A 49-year-old female was diagnosed with an atypical B-lymphoproliferative disorder with peripheral blood, bone marrow and breast involvement in the Pitié-Salpêtrière hospital in October 1995. Spleen and lymph nodes were not involved. The immunophenotype of peripheral blood lymphocytes was CD22$^+$, FMC7$^+$, CD79b$^+$, CD23$^-$, CD5$^+$ and IgM/D κ high (Matutes' score 2).[13] Apart from CD5, no T-cell markers were expressed. According to current WHO criteria,[14] this case could be classified pleomorphic mantle cell lymphoma. After CHOP/CLL type treatment,[15] complete remission was observed until May 2000. The lymphocyte count progressively increased, again with the same phenotype and reached 35×10^9/L with associated anemia. The patient was treated with ESHAP, rituximab and autologous bone marrow transplantation[16] and achieved a second durable complete remission which still persists as of September 2007. Informed consent was obtained from the patient and the study was approved by the INSERM review board (October 2005).

Cytogenetic analysis

Chromosome studies using RHG and GTG banding techniques were performed on peripheral blood cells. Description of chromosomal changes followed the ISCN recommendations.[17] Metaphase FISH analysis was performed as previously described.[18]

Nucleic acid methods

RNA and DNA extraction and analyses were performed using standard protocols. Nylon membranes were Hybond N+ (Amersham, Les Ulis, France). Inverse-PCR was performed as previously described[19] using inverse primers as follows: external-REV GCAGTGAGTGAGAGGTCAGCA, external-FOR GACAGTGAATAATGGCCCTACA, internal-REV GGTCCAGTCAACTTCCTGCT, and internal-FOR GTTACATTGCACATGATGACTATA. PCR products were then sequenced. Classical PCR was then used to confirm location and sequence of the breakpoint (FOR GCAAATCAAGGTGGCAAGGA AND REV GCAGTGAGTGAGAGGTCAGCA).

Sequence analyses were performed locally or at *http://genome.ucsc.edu*.

A human NKX2-5 HA-tagged expression construct was obtained by PCR amplification of the entire open reading frame, was cloned into the vector pcDNA3, and verified by DNA sequencing.

For RT-PCR analyses, 2 μg of total RNA extracted from patients' mononuclear bone marrow cells or cell lines were used to synthesize cDNA using the Superscript II kit (Invitrogen, Cergy-Pontoise, France) and random hexamers. PCR amplifications were performed starting from 2 μL of cDNA template for 33 cycles consisting of 94°C for 1 min; 55°C for 30 secs., and 72°C for 30 secs., primers used were as follow: NKX2-5-FOR TCTATCCACGTGCCTACAGC; NKX2-5-REV TGGACGTGAGTTTCAGCACG.

Probes for Southern blot

A probe corresponding to chromosome 5 sequences upstream of NKX2-5, spanning nucleotides 172596357 to 172598356, was obtained by PCR amplification of BAC 281H14 DNA using AAAGACACAGCTCCCGCAGGC and ACGAAGAGCAGAGTCGCGCT primers. The J δ1 probe is a 1.7 kb XbaI fragment spanning nucleotides 21988328 to 21990065 of chromosome.[14]

Western blot

50 μg of total cellular extracts were separated in 10% SDS-PAGE gel and then blotted to nitrocellulose membranes (Schleicher & Schuell, Cassel, Germany). 1:1,000 Anti NKX2-5 (SC-14033, Santa Cruz, Palo Alto, CA, USA) and 1:2,000 anti-actin (A5316, Sigma Aldrich, St-Quentin-Fallavier, France) antibodies were used.

Results and Discussion

Characterization of the chromosomal translocation

At diagnosis in 1995 (P-1), the karyotype was reported as 46,XX,t(5;14)(q34;q11)[23]/46, idem, i(8)(q10)[1]/46, XX[63] (Figure 1A). This translocation was present during all the disease course. In 2002 (P-2) the karyotype was 46,XX,t(5;14)(q34;q11)[5]/46,idem,t(15;21)(p11;p11)[3]/47,idem,+mar1[2]/44-46,XX,t(5;14)(q34;q11),-6,i(8)(q10),-9,add(11)(q24),+mar2[cp8]/46,XX[2]. FISH studies with IGH-CCDN1 probes ruled out a cryptic t(11;14)(q13;q32). In keeping with the location of the breakpoint, FISH analyses demonstrated split of *TCR* δ encompassing probes (Figure 1B). The use of FISH probes aimed at detecting the rearrangement of the *TLX3* locus on chromosome 5 resulting from the common t(5;14) translocation showed signals on both normal and rearranged chromosomes 5, indicating that the breakpoint was telomeric to this locus (*data not shown*). To check for involvement in the *NKX2-5* gene, we used BAC 466H21 as a FISH probe. This probe generated a signal on the der(5) and der(14) chromosomes in addition to a signal on the normal chromosome 5 (Figure 1B). Together, these data indicate that the *TCRD* gene was rearranged within the NKX2-5 locus as a result of this t(5;14) translocation. To establish involvement of the *TCRD* gene at the molecular level, we used a probe encompassing the J δ 1 segment in Southern blotting experiments. This probe identified an abnormal fragment in addition to the germline band in DNA digested with either *Bam*HI or *Bgl*II (Figure 2A). The same

rearrangement was observed at two different stages of the disease (P-1 and P-2). To better characterize the breakpoint on chromosome 5, we used an inverse PCR strategy starting from *TCR* sequences using DNA from P-1. Analyses of the nucleotide sequence of the amplified fragment located the breakpoint on chromosome 5, 2.5 kb downstream from the *NKX2-5* gene (see *Online Supplementary Figure 1* for a scheme of the loci). To confirm our result, we selected a probe from this region for Southern blot analyses of the patient's DNA. The probe detected an abnormal fragment upon digestion with EcoRI in addition to the germline band. This probe also identified an abnormal BglII fragment of similar size to the abnormal fragment detected by the J δ 1 probe (*data not shown*). To further compare the two stages of disease, the nucleotide sequence of the breakpoint was determined after PCR amplification of the patient's DNA and was shown to be identical. Nucleotide sequence alignment of the chromosomal breakpoint with its normal counterpart is shown in Figure 2C. This allowed us to locate the breakpoint on chromosome 14 immediately 5' of the D δ 3 segment. The sequence of the chromoso-

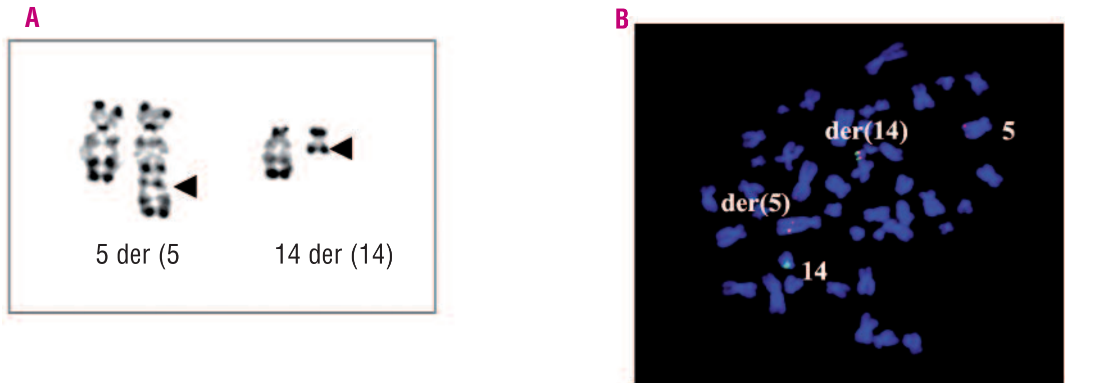

Figure 1. Cytogenetic analysis of patient P-1 (A) Partial karyotype of the patient (B) FISH analysis of P-1 metaphase. BAC probes are as follows RP11-466H21 (NKX2-5, red) and RP11-262M15 (TCRD, green).

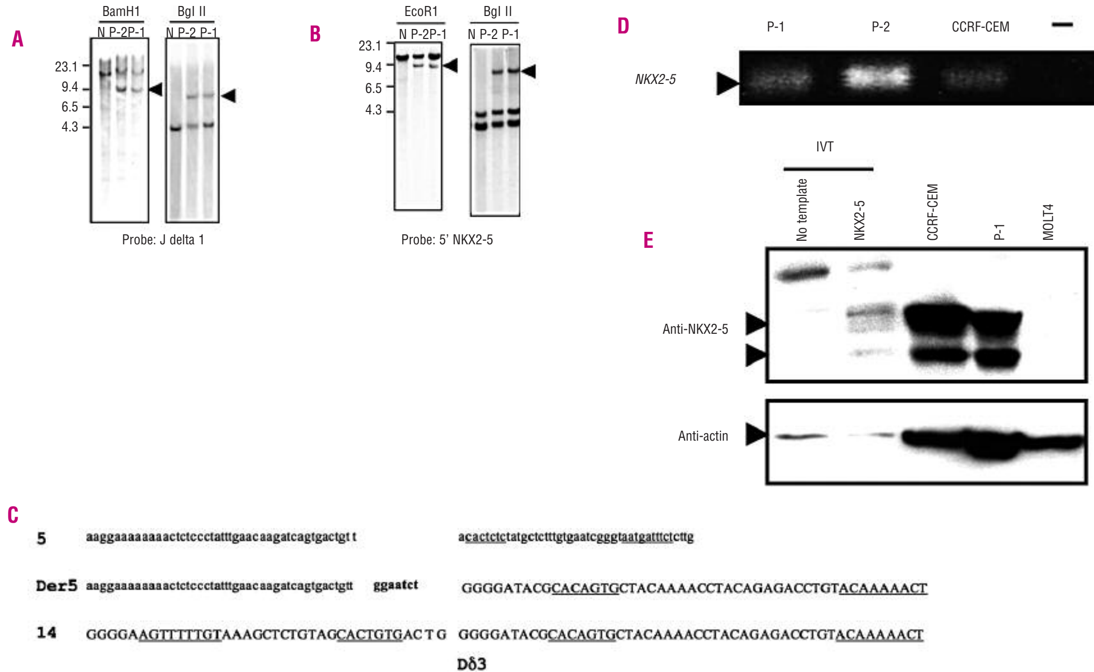

Figure 2. Analyses of the t(5;14) in patient P (A) Southern blot analyses of patient DNA for TCR δ gene status. A probe corresponding to the Jδ1 segment reacts with an abnormal fragment (indicated by an arrowhead) in patient's material with respect to a wild type control (N). Note that the same rearranged fragments are detected at both stages of the disease (P-1, P-2). (B) Southern blot analyses of patient DNA for NKX2-5 gene status. An identical rearrangement (indicated by an arrowhead) is also detected with a chromosome 5 probe at both stages of the disease. (C) Alignment of the t(5;14) fusion sequences and wild type counterparts. N nucleotides appear in bold lowercase letters. Recombination signals (RS) from Dδ3 segment and cryptic RS sequences from chromosome 5 are underlined. (D) RT-PCR analyses of NKX2-5. A specific fragment was amplified from cDNA from patient's material and in positive controls. (E) Western blotting analyses of NKX2-5 in patient sample. The NKX2-5 protein is detected as a doublet in patient material (P-1), as well as positive controls (IVT and CCRF-CEM). IVT: *in vitro* translation. Actin was used as a loading control. MOLT4 does not express NKX2-5. CCRF-CEM and MOLT4 are established human T-ALL cell lines used as positive and negative control respectively.

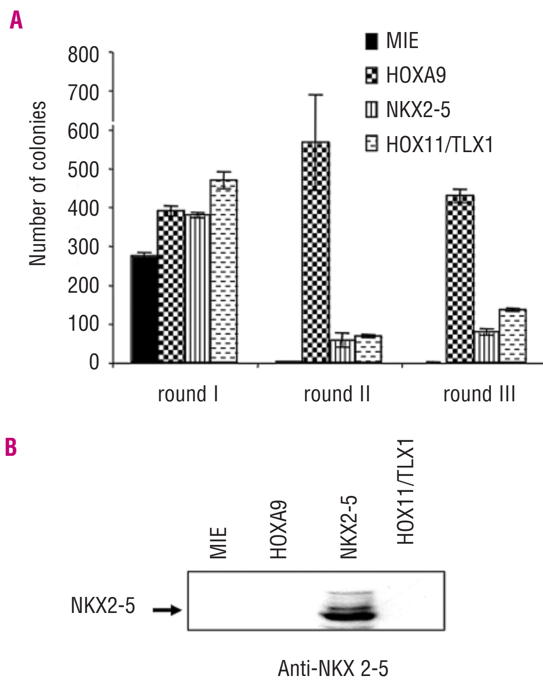

Figure 3 (*left*). Properties of myeloid precursors transduced with HOXA9, NKX2-5, TLX1. (A) Clonogenic properties of bone marrow cells upon serial replating on methylcellulose. Colony numbers are shown per 5×10³ cells. Results are average of 4 experiments. (B) Western blot of cell extracts from primary colonies from MIE (empty vector), *MSCV-HOXA9*, *MSCV-NKX2.5*, *MSCV-TLX1*-transduced cells. Equal amounts of protein were loaded and blots were probed with anti *NKX2-5*. Analyses of GFP expression and FACS analyses showing the myeloid nature of the transduced cells are shown in *Online Supplementary Figure 2*.

mal fusion demonstrated the presence of non-templated (N) nucleotides implicating V(D)J recombinase activity in the genesis of the translocation. On chromosome 5, recombination-related sequences, i.e. a canonical 21 bp spacer between heptamer and nonamer sequences, were observed which might account for the location of the chromosome 5 breakpoint.

RT-PCR analysis demonstrated the expression of *NKX2-5* at both stages of the disease, whereas this gene was not expressed in 41 B-cell lymphoma samples devoid of t(5;14) (Figure 2D and *data not shown*). NKX2-5 protein expression was confirmed in P-1. Transcriptional activation through *TCR*αδ gene rearrangement in a B-type neoplasm is a rare event.[20, 21] However, since the TCR enhancers are known to be lymphoid specific but not T-cell restricted, the functional consequences of these rearrangements are transcriptional activation of the partner genes.

To compare the biological properties of NKX2-5 to those of TLX1 and HOXA9, we used a myeloid colony formation assay. Murine hematopoietic precursors (Lin$^{neg/low}$) were transduced by MSCV based constructs expressing NKX2-5 and plated in methylcellulose. The colonies were scored and compared with empty viruses or viruses encoding HOXA9 or TLX1. All retroviruses were similarly efficient in transduction (*data not shown*). All three constructs conferred enhanced replating potential to the transduced cells (Figure 3A). The growth stimulatory effect towards myeloid progenitors was more pronounced for HOXA9 and TLX1, but NKX2-5 expression repeatedly conferred to myeloid progenitors the ability to generate colonies after the third round of replating, a feature never observed with empty vector. The moderate effect of *NKX2-5* markedly differs from the effect of HOXA9 and is comparable to the effect of TLX3 or HOXA13.[22] Homeobox proteins have documented effects on cell survival, proliferation and fate. The three orphan homeobox genes, *TLX1, TLX3* and *NKX2-5*, are known to be transcriptionally activated in T-ALL. During development, TLX1 is involved in genesis of the spleen, and *TLX1* and *TLX3* play a role in cellular fate determination of some neuron subtypes.[23] The cardiac homeobox protein, NKX2-5, is essential to cardiac development and mutations of NKX2-5 cause various congenital heart diseases.[24] It would be of interest to identify and compare the oncogenic pathways triggered by those oncoproteins. Ectopic expression of NKX2-5 has also been reported in two T-cell lines derived from T-cell malignant diseases and in a T-ALL patient.[12] This report also supports an oncogenic role for NKX2-5. The frequency of HOX gene activation in malignant B-cell diseases must still be determined, keeping in mind that our patient with a t(5;14) translocation exhibited an unusual extranodal disease.

Given the involvement of the TCR δ gene, the translocation might have occurred at an early step of lymphoid differentiation. Nevertheless, our results demonstrate that homeobox transcriptional activation occurs in both T- and B-lymphoid proliferation and also in both acute and chronic disease.

Authorship and Disclosures

XS designed research and perfomed research; VD-V perfomed research; ED provided vital reagents and revised the manuscript; ZA provided vital reagents; RB designed research and revised the manuscript; HM-B provided vital reagents and revised the manuscript; OAB funded research and revised the manuscript; FN-K designed research and wrote the manuscript.

The authors reported no potential conflicts of interest.

References

1. Boehm T, Rabbitts TH. The human T cell receptor genes are targets for chromosomal abnormalities in T cell tumors. Faseb J 1989;3:2344-59.
2. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004;306:269-71.
3. Armstrong SA, Golub TR, Korsmeyer SJ. MLL-rearranged leukemias: insights from gene expression profiling. Semin Hematol 2003; 40:268-73.
4. Aspland SE, Bendall HH, Murre C. The role of E2A-PBX1 in leukemogenesis. Oncogene 2001;20:5708-17.
5. van Grotel M, Meijerink JP, Beverloo HB, Langerak AW, Buys-Gladdines

JG, Schneider P, et al. The outcome of molecular-cytogenetic subgroups in pediatric T-cell acute lymphoblastic leukemia: a retrospective study of patients treated according to DCOG or COALL protocols. Haematologica 2006;91:1212-21.
6. Ballerini P, Blaise A, Busson-Le Coniat M, Su XY, Zucman-Rossi J, Adam M, et al. HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis. Blood 2002;100:991-7.
7. Berger R, Dastugue N, Busson M, Van Den Akker J, Perot C, Ballerini P, et al. t(5;14)/HOX11L2-positive T-cell acute lymphoblastic leukemia. A collaborative study of the Groupe Francais de Cytogenetique Hematologique (GFCH). Leukemia 2003;17: 1851-7.
8. Hansen-Hagge TE, Schafer M, Kiyoi H, Morris SW, Whitlock JA, Koch P, et al. Disruption of the RanBP17/ Hox11L2 region by recombination with the TCRd locus in acute lymphoblastic leukemias with t(5;14) (q34;q11). Leukemia 2002;16:2205-12.
9. Bernard OA, Busson-LeConiat M, Ballerini P, Mauchauffe M, Della Valle V, Monni R, et al. A new recurrent and specific cryptic translocation, t(5;14)(q35;q32), is associated with expression of the Hox11L2 gene in T acute lymphoblastic leukemia. Leukemia 2001;15:1495-504.
10. Su XY, Della-Valle V, Andre-Schmutz I, Lemercier C, Radford-Weiss I, Ballerini P, et al. HOX11L2/TLX3 is transcriptionally activated through T-cell regulatory elements downstream of BCL11B as a result of the t(5;14)(q35;q32). Blood 2006;108: 4198-201.
11. Nagel S, Kaufmann M, Drexler HG, MacLeod RA. The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14) (q35.1;q32.2). Cancer Res 2003;63: 5329-34.
12. Przybylski GK, Dik WA, Grabarczyk P, Wanzeck J, Chudobska P, Jankowski K, et al. The effect of a novel recombination between the homeobox gene NKX2-5 and the TRD locus in T-cell acute lymphoblastic leukemia on activation of the NKX2-5 gene. Haematologica 2006;91:317-21.
13. Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houlihan A, Que TH, Catovsky D. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 1994;8:1640-5.
14. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. The World Health Organization classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November, 1997. Hematol J 2000;1:53-66.
15. Leporrier M, Chevret S, Cazin B, Boudjerra N, Feugier P, Desablens B, et al. Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. Blood 2001;98:2319-25.
16. Sutton L, Maloum K, Gonzalez H, Zouabi H, Azar N, Boccaccio C, et al. Autologous hematopoietic stem cell transplantation as salvage treatment for advanced B cell chronic lymphocytic leukemia. Leukemia 1998;12: 1699-707.
17. ISCN. An International System for Human Cytogenetic Nomeclature. LG Shaffer and N Tommerup (ed); S. Karger, Basel, 2005.
18. Romana SP, Cherif D, Le Coniat M, Derre J, Flexor MA, Berger R. In situ hybridization to interphase nuclei in acute leukemia. Genes Chromosomes Cancer 1993;8:98-103.
19. Su XY, Busson M, Della Valle V, Ballerini P, Dastugue N, Talmant P, et al. Various types of rearrangements target TLX3 locus in T-cell acute lymphoblastic leukemia. Genes Chromosomes Cancer 2004;41:243-9.
20. Park JK, McKeithan TW, Le Beau MM, Bitter MA, Franklin WA, Rowley JD, et al. An (8;14)(q24;q11) translocation involving the T-cell receptor alpha-chain gene and the MYC oncogene 3' region in a B-cell lymphoma. Genes Chromosomes Cancer 1989;1:15-22.
21. Duro D, Bernard O, Della Valle V, Leblanc T, Berger R, Larsen CJ. Inactivation of the P16INK4/MTS1 gene by a chromosome translocation t(9;14)(p21-22;q11) in an acute lymphoblastic leukemia of B-cell type. Cancer Res 1996;56:848-54.
22. Su X, Drabkin H, Clappier E, Morgado E, Busson M, Romana S, et al. Transforming potential of the T-cell acute lymphoblastic leukemia-associated homeobox genes HOXA13, TLX1, and TLX3. Genes Chromosomes Cancer 2006;45:846-55.
23. Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, et al. Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci 2004;7:510-7.
24. Akazawa H, Komuro I. Too much Csx/Nkx2-5 is as bad as too little? J Mol Cell Cardiol 2003;35:227-9.