[haematologica]
2004;89:671-678

Two dual-color split signal fluorescence *in situ* hybridization assays to detect t(5;14) involving *HOX11L2* or *CSX* in T-cell acute lymphoblastic leukemia

LAURA J.C.M. VAN ZUTVEN
SANDRA C.J.M. VELTHUIZEN
INGRID L.M. WOLVERS-TETTERO
JACQUES J.M. VAN DONGEN
TIM S. POULSEN
RODERICK A.F. MACLEOD
H. BERNA BEVERLOO
ANTON W. LANGERAK

A B S T R A C T

Background and Objectives. The t(5;14)(q35;q32) is a novel cryptic translocation in pediatric T-cell acute lymphoblastic leukemia (T-ALL), involving *HOX11L2* or *CSX* on 5q35. The 14q32 breakpoints are heterogeneous. Because the t(5;14)(q35;q32) is hard to detect using conventional karyotyping, it is easily missed in routine diagnostics. Here we describe the development and application of split signal fluorescence *in situ* hybridization (FISH) assays for both *HOX11L2* and *CSX*, for detection of t(5;14) possibly present in T-ALL patients.

Design and Methods. We developed and validated two split signal FISH assays for metaphase and interphase detection of t(5;14) in T-ALL patients. We also investigated the involvement of *IGH* on 14q32. In addition, *HOX11L2* and *SIL-TAL1* expression was studied using reverse transcription polymerase chain reaction (RT-PCR).

Results. The FISH assays were validated on cell lines and T-ALL patients. We did not identify cases with a t(5;14)(q35;q32) involving *CSX*, but we did identify 5 cases of t(5;14) involving *HOX11L2* out of 32 T-ALL cases studied; in each case the 14q32 breakpoint was found to be centromeric to the *IGH* region. All 5 positive cases showed *HOX11L2* expression, as did 1 case without t(5;14)(q35;q32). Cases with t(5;14)(q35;q32) involving *HOX11L2* did not show *TAL1* abnormalities, whereas 5 *HOX11L2* negative cases did.

Interpretation and Conclusions. Using the newly developed and validated FISH probe sets, we identified 5 new cases of t(5;14) involving *HOX11L2* both on metaphases and interphases. The incidence of the t(5;14)(q35;q32) involving *CSX* is probably low. RT-PCR results suggest that *TAL1* and *HOX11L2* expression, or *TAL1* aberrations and the t(5;14)(q35;q32) involving *HOX11L2* are mutually exclusive.

Keywords: T-ALL, t(5;14), FISH, *HOX11L2*, *CSX*.

From the Department of Genetics (LJCMvZ, SCJMV), Erasmus MC, Rotterdam, The Netherlands; Department of Immunology, Erasmus MC, Rotterdam, The Netherlands (ILMWT, JJMvD, AWL); Department of Probe Application, DakoCytomation, Glostrup, Denmark (TSP); Department of Human and Animal Cell Culture, DSMZ, Braunschweig, Germany (RAFML); Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands (HBB).

Correspondence:
Anton W. Langerak, Department of Immunology, Dr. Molewaterplein 50, 3015 GE, Rotterdam, The Netherlands.
E-mail: a.langerak@erasmusmc.nl

Acute lymphoblastic leukemia (ALL) is a worldwide disease, with an incidence of approximately 6 cases per 100,000 inhabitants per year. Chromosomal rearrangements are found in many cases of ALL. Some of these rearrangements are specifically associated with certain subtypes of ALL. The presence of certain chromosome rearrangements constitutes an important prognostic factor for the outcome of disease and has therapeutic consequences.

In up to 30% of patients with T-cell acute lymphoblastic leukemia (T-ALL), aberrations of the *TAL1* gene (1p32) are found.[1] The majority are submicroscopic deletions of all coding exons of the *SIL* gene and the 5' untranslated region of *TAL1*. As a result, the *TAL1* coding region is placed directly under the control of the *SIL* promoter, leading to ectopic *TAL1* expression in precursor T-cells.[2-5] In another 3% of T-ALL patients ectopic *TAL1* expression is caused by translocations involving the 5' untranslated region of the *TAL1* gene.[3,6-9]

A novel cryptic translocation occurring mainly in childhood T-ALL is the recently described t(5;14)(q35;q32).[10,11] Fluorescence *in situ* hybridization (FISH) demonstrated that the 5q breakpoint is heterogeneous. In the majority of patients the breakpoint is located within or downstream of *RANBP17*. This locus is also involved in the t(5;14)(q35;q11), with the breakpoint on 14q11 involving *TCRD*.[12] A second breakpoint on 5q35, located 2 Mb telomeric of *RANBP17*, was recently found in the T-ALL cell lines PEER and CCRF-CEM immediately upstream of a homeobox gene, *NKX2-5* or *CSX*.[13] Thus far, the breakpoints on chromosome 14q seem to be very heterogeneous as well. Bernard *et al.*[10] and Hélias *et al.*[11] found that the 14q breakpoint was centromeric to the

IGH region. Additionally, Bernard *et al.*[10] narrowed the breakpoint region down to 700 kb between the *TCL1* and *AKT1* loci. No single clone encompassing the breakpoints in all patients tested has so far been found; however, the breakpoints in the patients' samples were all shown to be downstream of *BCL11B*,[10] thus resembling the location of the 14q32 breakpoints in the cell lines *HPB-ALL*,[14] CCRF-CEM and PEER.[13] *BCL11B* lies approximately 6.6 Mb centromeric of *IGH*.

The *RANBP17* gene does not seem to be deregulated as a result of the t(5;14)(q35;q32). However, another gene in the near vicinity, *HOX11L2* (also called *TLX3*), was ectopically expressed in these patients,[15] and in the pediatric T-ALL cell line HPB-ALL, which carries the same translocation.[14] Ferrando *et al.*[16] and Ballerini *et al.*[17] showed that *HOX11L2* expression is associated with a poor prognosis. However, Cavé *et al.*[18] recently showed that patients with *HOX11L2* expression did not have a significantly different clinical outcome from patients without this expression.

Because the t(5;14)(q35;q32) is cryptic and thus hard to detect using conventional karyotyping, it escapes detection by routine diagnostics. Therefore the incidence is underestimated. Here we describe the development and application of two dual-color split signal FISH assays for detection of t(5;14)(q35;q32) involving *HOX11L2* or *CSX*. We included the *CSX* variant, very recently described in T-ALL cell lines,[13] to investigate whether this variant also occurs in primary samples. The probe sets were validated in a series of 32 T-ALL cases. To further study additional heterogeneity of the 14q32 breakpoint in our cases, we used a split signal probe combination for the possible involvement of *IGH*, which is a well-known partner gene in many different translocations occurring in acute leukemia. We also investigated whether the t(5;14) involving *HOX11L2* or *CSX* and *TAL1* aberrations can co-exist.

Design and Methods

Cell lines and patient samples

The cell lines PEER, DU.528, CCRF-CEM, MOLT16, and HPB-ALL, established from patients with pediatric T-ALL, were obtained from the DSMZ (Braunschweig, Germany) (details given in Drexler[19] and at *www.dsmz.de*). They were cultured according to standard protocols, and cytogenetically prepared as described elsewhere.[20] The published karyotype, modified after FISH, for CCRF-CEM[13] is 46,XX, der(5)t(5;14)(q35.1;q32.2), t(8;9)(p11;p24),der(9)del(9)(p24)del(9)(q11q13~21), ins(14;5)(q32.2;q35.1q35.1), +20. The cell line used in this study had become near-tetraploid, as was also reported by Drexler.[19] This tetraploidization was also observed for HPB-ALL. Bone marrow or blood from 32 T-ALL patients was obtained at diagnosis and cultured and harvested using standard cytogenetic protocols.

For each patient 20-32 metaphase cells were analyzed using both Q- and R-banding. The chromosome aberrations observed were described according to the International System for Human Cytogenetic Nomenclature (ISCN) (ISCN, 1995). The patients' karyotypes are shown in Table 1. Methanol/acetic acid fixed cell suspensions were stored at –20°C. At diagnosis, informed consent was obtained from the patients and/or parents/guardians to use left-over diagnostic material for research purposes.

HOX11L2, CSX and TAL1 specific split signal probes

End-sequenced bacterial artificial chromosome (BAC) clones for the *HOX11L2* and *CSX* loci were identified by analysis of contig sequences using the TIGR STC-BAC program *http://www.tigr.org/tdb/humgen/bac_end_search/bac_end_search.html* and the UCSC Genome Bio informatics website *http://genome.ucsc.edu*. The DNA sequence of the different loci was assembled using Lasergene (DNASTAR inc. Madison, USA). For *HOX11L2* sequences AC021077, AC016574, AC010306, AC091980, AC093246, AC011400, and AC022426 were used; for *CSX*, the published sequences AC110011, AC008429, AC008378, AC008412, AC106731, AC016573, AC008632, AC008663, AC010339, AC008674, and AC093275 were used. These analyses identified BAC clones with 5'- and 3'-end-sequences >200 bp in length displaying > 94% identity.

For the t(5;14)(q35;q32) involving *HOX11L2*, breakpoints on the der(5)t(5;14) have been described between *RANBP17* exon 20 and *HOX11L2* exon 1, while one T-ALL patient had a translocation breakpoint downstream of V *HOX11L2* exon 1.[10] These results were used to design a split signal FISH assay (DakoCytomation, Glostrup, Denmark) for translocations involving *HOX11L2* by selecting one BAC clone located < 1 kb centromeric to *RANBP17* exon 19 which spans 192 kb of DNA (HOX11L2-U) and three BAC clones spanning 269 kb located telomeric to the breakpoint region (HOX11L2-D) (Figure 1A). A 121 kb gap separates the HOX11L2-D and HOX11L2 probes. For the t(5;14)(q35;q32) involving *CSX*, which is transcribed in a telomeric-centromeric direction, the described breakpoints on the der(5) mapped upstream of *CSX* exon 1 in the PEER and CCRF-CEM cell lines.[13] Analysis of BAC clones telomeric of these breakpoints revealed 3 partially overlapping BAC clones spanning 523 kb (CSX-U), whereas analysis of the BAC end-sequenced clones centromeric to the breakpoint region revealed one clone with an end-sequence located 36 kb downstream of exon 2 of the *CSX* gene and an intermediate clone with

Table 1. Karyotypes, FISH and RT-PCR results of 32 cases of T-cell lymphoblastic leukemia (T-ALL) and 5 T-ALL cell lines.

Case/ cell line	Age(y) /sex	Karyotype	HOX11L2 FISH	CSX FISH	IGH FISH	HOX11L2 RT-PCR	TAL1 FISH#	SIL-TAL1 RT-PCR
1	18/F	46,XX[32]	Split	N	der(5)	+	N	–
2	5/M	46,XY,ins(5)(q3?p1?4p1?2)[15]/46,XY[4]	Split	der(14)	Metaphases N	+	Nd	–
3	6/M	Diagnosis: 46,XY,del(11)(q2?1q2?3)[21]/46,idem,add(9)(q11)[4] Relapse:46,XY,der(6)t(6;8)(q2?6;q24), del(8)(q2?4),del(9)(p21p21),der(9)del(9)(p21p21) add(9)(q11),del(11)(q2?1q2?3)[6]/46,idem,der(3;9)(p10;q10), der(6)t(3;6)(q2?9;p2?5), +del(9)[8]/46,XY[11]	Split	der(14)	der(5)	+	Nd	–
4	8/F	46,XX[61]	Split	der(14)	der(5)	+	N	–
5	14/M	46,XY,t(7;9)(p1?3;p2?2)[30]/46,XY[4]	Split	der(14)	der(5)	+	Nd	–
6	7/M	46,XY[74]	N	N	N	Nd	Nd	Nd
7	5/F	46,XX,t(5;16)(p10;q10)[8]/46,XX[15]	N	N	N	+	Nd	–
8	12/M	46,XY[22]	N	N	Nc	–	Nd	+
9	10/M	46,XY,del(9)(p13p23),t(11;14)(p12;q31)[14]/46,XY[10]	N	N	N	–	Nd	–
10	11/F	46,XX[20]	N	N	N	–	Nd	–
11	14/M	Diagnosis: 46,XY[26] Relapse: 46,XY[26]	N	N	N	–	Nd	–
12	9/M	46,XY[22]	N	N	N	Nd	Nd	Nd
13	2/M	46,XY,+10[20]/46,XY[7]	N	N	N	–	Nd	–
14	12/M	Diagnosis: 46,XY,t(1;13;9)(q2?4;q1?4;p2?1), add(7)(q22),der(10)t(7;10)(q22;p12),del(11) (q23q25), t(14;16)(q3?2;q2?3)[21]/46,XY[10]	Nd	Nd	Nd	Nd	Nd	Nd
		Relapse after BMT: 45,XY, t(1;13;9)(q2?1;q13;p2?1),add(3)(q13),add(7)(q22),der(10) t(7;10)(q22;p12), der(11)add(11)(p1?3)del(11)(q23q25),t(14;16)(q3?2;q2?3),add(17)(p11), 18,+1~2mar[cp7]/45,idem,-X,+t(X;12)(p10;p10),-Y,t(1;8)(p10;p10),add(3)(p21),add(4)(q2?6), add(7)(q22), der(9)t(1;13;9),+der(9)t(1;13;9)ins(9;?)(p2?1;?),-der(11),+add(11)(p15),add(11) (q14),-12,- der(13)t(1;13;9),+add(13)(q1?3),der(13)t(13;?;Y)(q13;?;q11),t(18;20)(p10;p10)[13]/46,XX(donor)[1]	N	N	N	Nd	Nd	–
15	12/M	47,XY,+mar1[9]/48,idem,+mar2[7]/46,XY[8]	N	N	N	–	Nd	–
16	2/M	46,XY[25]	N	N	N	–	Nd	–
17	20/M	45,XY,-7,der(9)t(7;9)(q11;p12~13)[14]/46,XY[11]	N	N	N	–	SIL deletion	+
18	16/F	46,XX,add(5)(q3),add(7)(q3?1),t(7;14)(p13;q32), del(11)(q13q23),del(12)(p1?3),add(15) (p1),del(18)(p11)[31]/46, XX[2]/after FISH: 46,XX,der(5)t(5;21),add(7)(q3?1),t(7;14) (p13;q32),del(11)(q13q23),del(12)(p1?2p1?3), der(15)t(12;15)(p1?3;p1),del(18)(p11)	N	Loss of 1 fused signal	der(7)	–	N	–
19	24/F	47-48,XX,?der(3),?der(11),del(12)(p1?1),+14,+21,+21, inc[cp11]/47-48,?der(3),?der(11),i(12)(q10),+21,+21,inc[cp8]/46,XX[1]	N	N	3 fused signals	– –	N	
20	10/F	45,XX,der(9)t(9;14)(p13;q1?2),del(12)(p12p13), -14,idic(17)(p11)[36]/46,XX[2]	N	N	N	–	SIL deletion	+
21	4/M	45,XY,del(9)(p13),der(13;14)(q10;q10)c[3]/45, idem,del(6)(q1?5q2?4)[13]/ 45,idem,del(6)(q?16q2?2)[10]/45,XY, der(13;14)(q10;q10)c[39]	N	N	der(13;14)c	–	Nd	–
22	33/M	44-46,Y,der(X)t(X;15)(p11;q1?)[16], del(5)(q23q34)[5],der(10)t(X;10)(p11;p11)[2],add(15) (q2?2)[6], del(17)(p11p13)[cp17]/46,XY[3]	N	N	N	–	Nd	Nd
23	71/F	46,XX,del(3)(q2?1q2?6)[9]/46,XX[12]	N	N	No metaphases	Nd	N	–
24	8/M	46,XY[32]	N	N	N	–	SIL deletion	+
25	8/F	46,XX,?add(9)(p1?)[6]/46,XX[30]	N	N	N	–	Nd	+
26	2/F	46,XX,t(6;11)(q27;q23)[10]/47,idem,+8[6]/46,XX[8]*	N	N	N	–	Nd	–
27	8/F	46,XX,add(2)(q1?),add(6)(q2?3),del(9)(q1?3~2?1q3?1), ?del(11)(q?),der(21)t(2;21) (q11~12;q22)[10]/46,XX[14]	N	N	N	–	Nd	–
28	12/M	Diagnosis: Nd, Relapse: 46,XY[34]	N	N	N	–	Nd	–
29	4/F	46,XX[28]	N	N	N	–	Nd	–
30	55/M	45,XY,add(2)(p2?3),add(7)(q21),-9,-15,-16, del(17)(p11),+mar1,+mar2[17]/ 46,XY[6]	N	N	N	–	Nd	–
31	14/M	46,XY,der(1)t(?;1)(?;p3?3)t(?;6)(?;p11),add(6)(p11), der(?)t(?;1)(?;p3?3)[9]/ after FISH: 46,XY,der(1)t(1;6) (p3?3;p11)ins(1;14)(p33;q11q32),der(6)t(6;14)(p11;q32), der(14)t(1;14) (p3?3;q11)/46,XY[16]	N	N	Split	–	Nd	–
32	9/M	46,XY,add(1)(p31)[2],del(6)(q2q2)[3],inc[cp5]/46,XY[18]	N	N	N	–	Nd	–
HPB-ALL		Karyotype according to literature**: 46,XY, der(1)t(1;16)(q22;p11~12)add(16)(p13),del(2) (p24),del(3)(p11), der(5)t(1;5)(q22;q32~33),r(16)(?p12?q12)/45,idem, -del(3),-3/94,idemx2, +del(21) (q12)x2/ after FISH***: der(1)(1pter→q22::16q22→q22::14q32.2→qter), del(2)(p24),del(3)(p11),der(5)(5pter→q35::1q22→qter),der(14)(pter→q32.2::5q35→5q35:: 16p11.2→p13.1:: 16q23→qter),der(16)r(16)(p11~q11q22)del(16)(q11.2q12~21)	Split	N	der(1), der(1)	+	N	Nd
PEER		Karyotype according to literature**: 42-47,XX,ider(4)(q10)del(4)(q2?8q3?1), del(5)(q22q31), del(6)(q13q22),del(9)(p11p22),del(9)(q22)/after FISH****: 46,XX,ider(4)(q10)del(4) (q2?8q3?1),der(5)del(5)(q22q31)t(5;14)(q35.1;q32.2),del(6)(q13q22), del(9)(p11p22),del(9) (q22),ins(14;5)(q32.2;q35.1q35.1)	N	CSX-U deletion	Nd	–	N	Nd
CCRF-CEM		Karyotype according to literature**: 88-101,XX,-X,-X,t(8;9)(p11;p24)x2, der(9)del(9)(p21~22) del(9)(q11q13~21)x2,+20,+20; sideline with +5,+21,add(13)(q3?3),del(16)(q12)/ after FISH****: 46,XX,t(8;9)(p11;p24),der(5)t(5;14)(q35.1;q32.2),der(9)del(9)(p24)del(9) (q11q13~21), ins(14;5)(q32.2;q35.1q35.1),+20	N	CSX-U deletion	Nd	–	*SIL* deletion	Nd
DU.528		Karyotype according to literature**: 46,XY,+del(1)(p33),+del(1)(q11), t(1;14)(p33;q11),del(13) (q14),-14	N	N	Nd	Nd	t(1;14) (p32;q11)	Nd
MOLT16		Karyotype according to literature**: 43-47,XX,t(3;11)(p21;p13), der(7)t(7;7)(qter→p15::q11.2→qter),t(8;14)(q24;q11),-9,dup(9)(pter→p13::p24→qter), -15,der(15)t(15;19)(qter→ p1?1::q1?1→qter)	N	N	Nd	–	*SIL* deletion	Nd

*M: male; F: female; age: age at diagnosis; N: normal hybridization pattern; Nd: not done; °a band of the expected size was observed in RT-PCR; - no band was observed in RT-PCR; Nc: non-conclusive result; # patients' TAL1 FISH described in Van der Burg et al.;[21] * t(6;11)(q27;q23) with MLL-AF6 fusion ** details given at www.dsmz.de/ and in Drexler[19]; ***recent karyotyping results described in MacLeod et al.[14] ****recent karyotyping results described in Nagel et al.[13]*

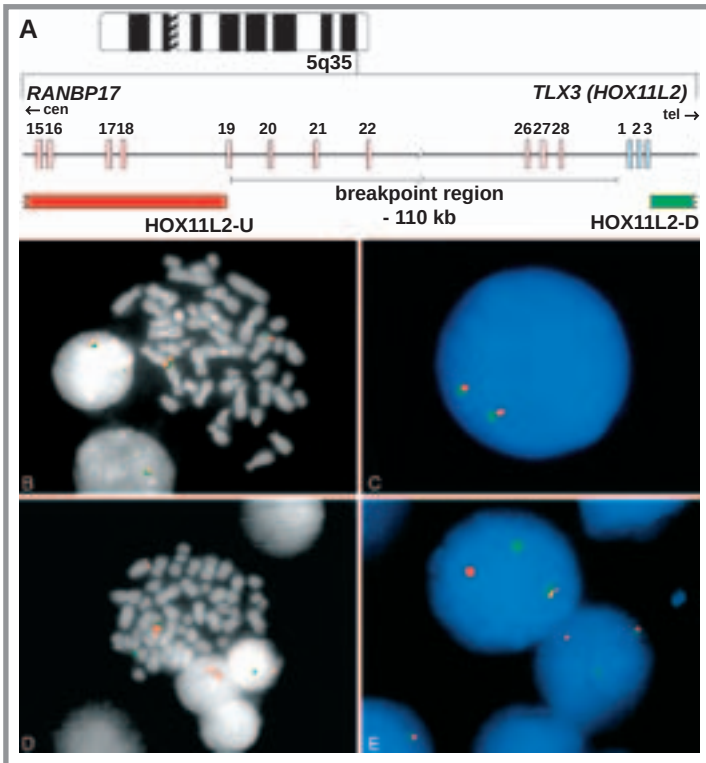

Figure 1. Split signal FISH using *HOX11L2* probes A) Schematic representation of part of the *RANBP17* and *HOX11L2* genes on chromosome region 5q35. The HOX11L2 translocation breakpoint region is indicated by the thin bar. The HOX11L2-U FISH probe consisted of one and the HOX11L2-D probe of three BAC clones. B) Metaphase spread of a healthy donor, depicted in inverted DAPI staining showing the co-localized signals on chromosomes 5. C) Interphase nucleus of a healthy donor, showing two fused signals of the normal chromosomes 5. D) Metaphase spread of t(5;14) (case 5), depicted in inverted DAPI staining showing one fused signal on the normal chromosome 5 as well as a separate green (HOX11L2-D) signal on the der(5) and red (HOX11L2-U) signal on the der(14). E) Interphase nucleus depicting t(5;14) (case 5), showing one fused signal (normal chromosome 5), as well as separate green (HOX11L2-D) and red (HOX11L2-U) signals.

a gap of less than 1 kb (CSX-D, covering 330 kb) (Figure 2A). The CSX-U and CSX-D probes (DakoCytomation, Glostrup, Denmark) are separated by a 117 kb gap. Both *HOX11L2* and *CSX* FISH probe sets are available from DakoCytomation (*Dr. T.S. Poulsen, Department of Probe Application, Produktionsvej 42, DK-2600, Glostrup, Denmark*).

For the investigated cell lines, the split signal FISH assay for *TAL1* aberrations was modified from that published by van der Burg *et al.*,[21] in that the downstream TAL1-D probe, a single PAC, was replaced by BAC clones comprising 566 kb (DakoCytomation, Glostrup, Denmark). The patients' *TAL1* FISH analysis was performed as described by van der Burg *et al.*[21] and results were described in that publication. All clones were further verified by end sequencing, restriction endonuclease digestion and FISH. As probes for the *IGH* locus on 14q32, α[22] (*kindly provided by Dr. H. Döhner, Heidelberg, Germany*) and IGH[23] (*kindly provided by Dr. H. Riethman, Wistar Institute, Philadelphia*) were used.

Fluorescence in situ hybridization

Growth of clones and DNA purification were performed according to the manufacturer's instructions using QIAGEN 500 tips (Qiagen, Hilden, Germany). Each probe set, designed to flank likely breakpoints, was labeled by nick translation with either Texas Red-dCTP (CSX-D, HOX11L2-U, SIL-U) or FITC-dCTP (CSX-U, HOX11L2-D, TAL1-D) and either biotin-16-dUTP (α) or digoxigenin-11-dUTP (IGH). Freshly prepared metaphase spreads from methanol/acetic acid cell suspensions stored at –20°C were used for FISH.

HOX11L2, *CSX* or *TAL1* probe mixtures consisted of 100 ng of each probe in 10 μL hybridization buffer (45% formamide, 300 mM NaCl, 5 mM phosphate, 10% dextransulphate, blocking PNA). Repetitive sequences were suppressed using blocking PNA (DakoCytomation, Glostrup, Denmark). Chromosomes and probes were denatured simultaneously for 5 min at 80°C, and hybridized overnight at 45°C in a moist chamber. The slides were stringently washed in 0.2× SSC/0.1% Triton X-100 at 65°C for 10 min, passed through a wash buffer (TBS) for 1 min, dehydrated through an ethanol series (70%, 85%, and 96%) and mounted with antifade containing 4'6-diamino-2-phenyl indol (DAPI) as counterstain.

For *IGH* split signal FISH, slides were pre-treated with RNAse and pepsin, and post-fixed with formaldehyde. Hybridization of 100 ng of each probe was performed overnight at 37°C in a moist chamber, and the slides were washed with 0.4×SSC at 72°C. Biotinylated probes were detected using FITC-labeled avidin, followed by biotinylated goat-anti-avidin and avidin-FITC incubation. Digoxigenin-labeled probes were detected using sheep-anti-digoxigenin-rhodamine, followed by donkey-anti-sheep-Texas Red incubation. Slides were counterstained with DAPI. For each sample a minimum of 100 interphase cells were scored, as well as 5-10 metaphases if present. Images were captured using a Zeiss epifluorescence microscope using MacProbe software (version 4.3, Applied Imaging, Newcastle upon

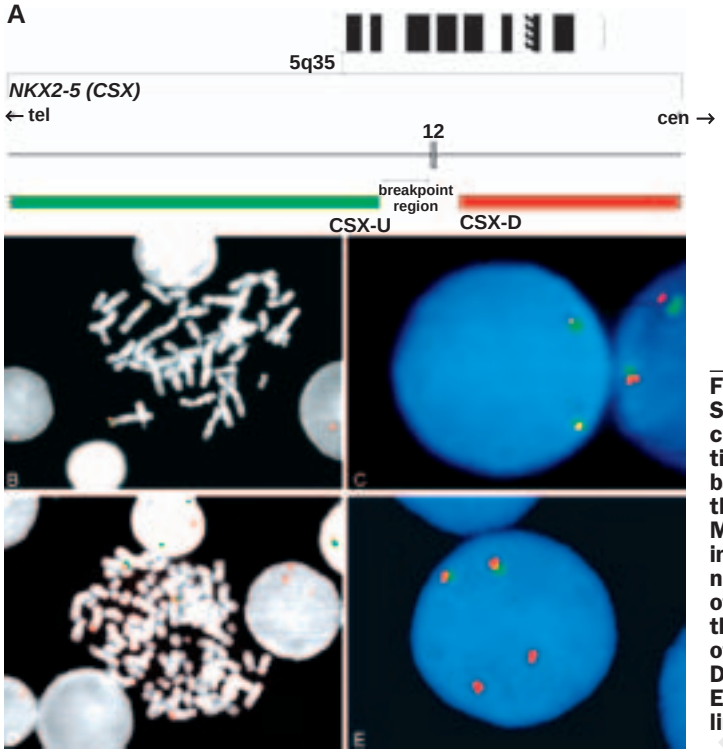

Figure 2. Split signal FISH using *CSX* probes A) Schematic representation of the *CSX* gene on chromosome region 5q35. The *CSX* translocation breakpoint region is indicated by the thin bar. The CSX-D FISH probe consisted of two and the CSX-U probe of three BAC clones. B) Metaphase spread of a healthy donor, depicted in inverted DAPI staining showing the fused signals on chromosomes 5. C) Interphase nucleus of a healthy donor, showing two fused signals of the normal chromosomes. D) Metaphase spread of the CCRF-CEM cell line, depicted in inverted DAPI staining showing the unbalanced t(5;14). E) Interphase nucleus of the CCRF-CEM cell line, showing the unbalanced t(5;14).

Tyne, UK).

Reverse transcription polymerase chain reaction

The presence of *SIL-TAL1* fusion transcripts was determined by RT-PCR as described elsewhere.[24] *HOX11L2* RT-PCR was performed using primers F-HOX11L2-EMC (5'-GGTTCCAAAACCGGAGGAC-3') and R-HOX11L2-EMC (5'-TGCAGACAGAGCGGGTCAG-3'), resulting in a 154 bp PCR product. The reaction mixture, consisting of 1×Gold buffer, 1 U TaqGold, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 10 pmol of each primer, was run on an ABI 9600 PCR machine (Applied Biosystems, Foster City, CA, USA) according to the following program: 7 minutes at 95°C, followed by 35 cycles consisting of 30 seconds at 94°C, 45 seconds at 60°C and 90 seconds at 72°C, with a final extension at 72°C for 10 minutes.

Results

Verification and validation of HOX11L2, CSX, and TAL1 probes

Verification of the clones using five different enzymes separately showed that the restriction endonuclease band patterns for all clones were identical to those predicted *in silico*. For *HOX11L2* and *CSX*, the BAC end-sequences of the clones showed >98% identity compared to the already published sequences at TIGR. Using the dual color split signal probes, FISH for *HOX11L2*, *CSX* and *TAL1* was subsequently tested on five T-ALL cell lines PEER, DU.528, CCRF-CEM, MOLT16, and HPB-ALL (Table 1). As expected, only HPB-ALL was positive for the *HOX11L2* translocation,[14] whereas the remaining cell lines were negative. PEER and CCRF-CEM (Figures 2D, 2E) showed an abnormal hybridization pattern using the *CSX* probe set.[13] These cell lines, both having an unbalanced der(5) t(5;14)(q35;q32) and a concomitant ins(14;5) (q32.2;q35.1q35.1),[13] showed a deletion of *CSX-U*. The remaining three cell lines were negative. DU.528 showed a t(1;14)(p32;q11),[19] while MOLT-16 and CCRF-CEM showed deletion of sequences between *SIL* and *TAL1*.[19] PEER and HPB-ALL showed no *TAL1* rearrangements by FISH.

To validate the new diagnostic probe sets further, the cut-off values were determined for each set. For this purpose, those cases without the translocation involving the gene studied were selected (n=16 for *HOX11L2*; n=36 for *CSX*). The cut-off values for each probe set were defined as the mean plus three times the standard deviation. This, for the split-signal pattern, resulted in cut-off values of 3% for *HOX11L2* and 2% for *CSX*. These values are far lower than all percentages observed in all positive cases. The cut-off values for loss of a complete fused signal were 4% for both probe sets. We did not observe deletion of either the green (HOX11L2-D or CSX-U) or the red (HOX11L2-U or CSX-D) signal in any of the cases without the t(5;14)(q35;q32).

Since these results showed that the new diagnostic probe sets for *HOX11L2* and *CSX* hybridize to the expected chromosomal localization (Figures 1B and 2B) and

demonstrated a split signal in both interphases and metaphases in case of a translocation (Figures 1D, 1E, 2D, and 2E) in the cell lines, the probes were used to analyze 32 patients, most of whom were children, with T-ALL.

Cytogenetics and t(5;14) FISH results of 32 T-ALL cases

The patients' karyotypes and FISH results for the different t(5;14) split signal assays are shown in Table 1. Using conventional cytogenetics, 11/32 cases were normal while the remaining 21 cases showed abnormal karyotypes, 9 of which were complex (>3 structural aberrations). All cases were tested for the presence of the t(5;14) using the newly developed probe combinations. Using the *HOX11L2* probe combination, both metaphase and interphase nuclei from 5 out of 32 cases (cases 1-5) showed one co-localized HOX11L2-D and HOX11L2-U signal, as well as a separate green (HOX11L2-D) and a separate red signal (HOX11L2-U), indicating a break in this region (Figures 1D, 1E). In the metaphases, the fused signal was located on the normal chromosome 5; the HOX11L2-U signal was visible on the der(5), whereas the HOX11L2-D signal was translocated to the der(14) (Figure 1D).

When investigating all cases with the split signal probe combination for *CSX*, no translocations involving *CSX* were found. In metaphases of cases 2, 3, 4 and 5 (positive for split HOX11L2 signals) one co-localized CSX-D and CSX-U signal was observed on the normal chromosome 5, whereas the other fused signal was translocated to the der(14). These results are consistent with the fact that *HOX11L2* is located centromeric to *CSX*. This change could of course not be observed in interphase nuclei, since the *CSX* signals do not split due to the centromeric breakpoint in *HOX11L2*. In case 1, no metaphases were present to confirm translocation of the fused *CSX* signal to the der(14). In case 18, we observed loss of one fused *CSX* signal, which can probably be explained by the presence of an unbalanced t(5;21) observed in the karyotype after FISH and SKY.

Next, we also analyzed the 14q32 breakpoint using probes for the *IGH* gene (Table 1). In 1 of 32 cases (case 31), an abnormal hybridization pattern was found in interphase nuclei. This case showed one fused signal as well as one separate green (α) and red (IGH) signal, indicating a break in the *IGH* gene. Additionally, in 4 of the 5 cases showing the split signal for *HOX11L2* (cases 1, 3, 4 and 5), one fused α and IGH probe signal was visible on the der(5), indicating that the 14q breakpoint in these t(5;14) cases is indeed centromeric to *IGH*. Case 18 showed two fused signals, one on chromosome 14 and one on a der(7)t(7;14), whereas case 21 showed fused signals, one on chromosome 14 and one on the Robertsonian der(13;14)(q10;q10). Finally, case 19 showed 3 co-localized signals on 3 copies of chromosome 14. For case 2, which showed the separate HOX11L2-D and HOX11L2-U signals and the *CSX* fused signal on a der(14), the metaphases found in the slide hybridized for *IGH* showed fused signals on chromosomes 14. Probably, the percentage of abnormal metaphases in this slide was too low to detect a metaphase with the t(5;14), although we cannot fully exclude the possibility that the 14q32 breakpoint is telomeric to the *IGH* gene.

HOX11L2 and SIL-TAL1 expression

From 28/32 cases sufficient material was available to investigate *HOX11L2* expression. Six cases, among which all 5 of the t(5;14)+ cases (cases 1-5, Table 1), showed a transcript using RT-PCR; the other 22 cases were negative. The sixth PCR positive case was case 7, in which no translocation was detected with the split signal *HOX11L2* and *CSX* probe sets.

Ferrando *et al.*[16] showed that expression of *TAL1* and *HOX11* (to which *HOX11L2* is closely related) are mutually exclusive within leukemic samples. Furthermore, analysis of expression in the human T-ALL cell lines used here indicated that the presence of a *SIL-TAL1* fusion might be a negative predictor for the presence of the t(5;14)(q35;q32) involving *HOX11L2*. In the CCRF-CEM cell line, both a *SIL-TAL1* fusion and a t(5;14)(q35;q32) involving CSX did occur. If sufficient material was available, *SIL-TAL1* fusions had been searched for at diagnosis, using RT-PCR on a routine basis, and/or FISH. cDNA of 29 cases was available for *SIL-TAL1* RT-PCR (Table 1); 5 cases (8, 17, 20, 24 and 25) showed a fusion between *SIL* and *TAL1*, 3 of which (17, 20 and 24) also showed an abnormal FISH hybridization pattern using the *TAL1* probe set (Table 1). Of the other 2 cases (case 8 and 25) no methanol/acetic acid fixed cell suspension was available for additional *TAL1* FISH analysis. All 5 cases were negative for the t(5;14) involving *HOX11L2*, indicating that *SIL-TAL1* fusions and the t(5;14) involving *HOX11L2* are mutually exclusive. Unfortunately, as no patients with *CSX* involvement were identified, we could not draw definite conclusions about the co-existence of *CSX* and *TAL1* aberrations.

Discussion

Here we describe two new dual-color split signal FISH assays for the detection of t(5;14)(q35;q32) involving *HOX11L2* or *CSX*, or other translocations involving these genes, e.g. t(5;14)(q35;q11). These t(5;14)(q35;q32) are cryptic translocations and thus may escape detection using routine diagnostic methods even when augmented by chromosome painting. Single locus FISH is a helpful technique to identify juxtapositional

rearrangements and gene fusions with variable or widely displaced breakpoints or partner genes, which may be laborious to detect using molecular methods such as RT-PCR. The wide dispersal of 14q32 breakpoints in the far downstream region of *BCL11B*, together with its inconsistent expression, effectively rule out PCR-based methods for detecting t(5;14).

The split signal FISH assays we developed for *HOX11L2* and *CSX* should fill a niche in identifying these translocations in new patients. The assays were first validated on T-ALL cell lines known to have a t(5;14)(q35;q32). The cut-off values calculated for the 2 probe sets were 3% for *HOX11L2* and 2% for *CSX*. Using these newly developed diagnostic sets, 32 T-ALL cases were analyzed for the presence of both types of t(5;14) involving either *HOX11L2* or *CSX*. To study the breakpoint on chromosome 14q32, a split signal FISH assay for *IGH* was also used. We identified 5 cases with a t(5;14) involving *HOX11L2*; conventional karyotyping had not shown this translocation. In 3 of our 5 cases, (complex) chromosomal aberrations were observed, indicating that the t(5;14) does occur when other abnormalities are present. For the t(5;14) involving *HOX11L2*, we observed an incidence of 15% in our total series and 19% (5/27) in childhood and adolescent T-ALL. These percentages resemble those published by Bernard *et al*. (16.7 and 22%, respectively).[10] Direct comparison with data from Berger *et al*.[25] and Cavé *et al*.[18] is impossible as these researchers pooled their t(5;14) and *HOX11L2*-expressing cases. However, unlike Berger *et al*.,[25] we did not observe the t(5;14) in the 3 adult patients we investigated. We did not identify any cases of a t(5;14)(q35;q32) involving *CSX*, indicating that the incidence of this alternative 5q35 translocation is probably low. However, at this point we cannot exclude the possibility that this variant has its origin in culture. The 14q breakpoint was found centromeric to the *IGH* locus in our groups of patients; in 4 of 5 t(5;14) cases, the fused α and IGH probe signal translocated to the derivative chromosome 5. These results confirm the findings of Bernard *et al*.[10] and Hélias *et al*.[11] Therefore, studying the region downstream of *BCL11B* in our t(5;14) positive cases in more detail could help to delineate the 14q32 breakpoint more precisely. Activation of *HOX11L2* expression through these different 14q loci could contribute to leukemic transformation of normal bone marrow cells, e.g. through regulatory elements of *BCL11B* – a gene which has recently been shown to control thymocyte development and survival;[26] another possibility, however, is that disruption of negative regulatory regions at 5q35 (*HOX11L2* is not expressed during T-cell development) mediates this effect. The hypothesis which best explains the extraordinary recurrence of t(5;14)(q35;q32) in T-ALL is ectopic activation of *HOX11L2* following juxtaposition with regulatory elements present in the far downstream region of *BCL11B*. We did not observe the t(5;14)(q35;q11) in our series. However, the probe set used here would detect this translocation as well, as the 5q breakpoint described for these cases is situated within the same region as for the t(5;14)(q35;q32) involving *HOX11L2*.[12]

It has been shown that cases with a t(5;14)(q35;q32) involving *HOX11L2* exhibit *HOX11L2* expression[10,14] whereas precursor B-ALL, adult T-ALL and 40% of childhood T-ALL cases mostly do not.[15] However, more recently t(5;14)(q35;q32) and/or *HOX11L2* expression was observed in 13% of adult T-ALL cases by Berger *et al*.[25] Our 5 cases having the t(5;14) involving *HOX11L2* did show *HOX11L2* expression. Additionally, we observed *HOX11L2* expression in one case without the t(5;14) by FISH, showing that the presence of *HOX11L2* expression does not always require the presence of a t(5;14)(q35;q32). Mauvieux *et al*.[15] also observed this in one of their cases. Therefore FISH is needed to make an accurate diagnosis of t(5;14)(q35;q32). In the first instance, an unexplained correlation of *HOX11L2* expression with male sex was observed by Mauvieux *et al*.[15] They observed *HOX11L2* expression only in male t(5;14)(q35;q32) cases, whereas only 1 female case with *HOX11L2* expression was observed by others[10] until recently. In our series, we identified 5 new t(5;14) cases, including 2 female cases, both showing *HOX11L2* expression. Berger *et al*.[25] identified 67 t(5;14)/*HOX11L2* positive cases (defined as cases demonstrating either a 5q35 breakpoint involving *HOX11L2* (47 cases) by FISH or *HOX11L2* expression (55 cases) by RT-PCR). They did not observe any male preference in their series. However, direct comparison with their data is difficult as they pooled the translocated and *HOX11L2*-expressing patients. This also holds true for the 35 t(5;14)/*HOX11L2*-positive cases, including 7 females, described by Cavé *et al*.[18]

Expression of *TAL1* and *HOX11* (to which *HOX11L2* is closely related) has been shown to be mutually exclusive within leukemic samples and expression of these genes may be used to stratify different prognostic subgroups.[16] Furthermore, analysis of human T-ALL cell lines indicated that the presence of *TAL1* aberrations might be a negative predictor for the presence of the t(5;14)(q35;q32) involving *HOX11L2*. In our series, no cases having the t(5;14), involving *HOX11L2*, showed *TAL1* abnormalities, whereas 5 other cases were positive for the *SIL-TAL1* fusion product. These results suggest that *TAL1* expression and *HOX11L2* expression, and therefore also *TAL1* expression and the t(5;14)(q35;q32) are very unlikely to co-exist in one patient. This is in line with similar findings in the very recent studies by Berger *et al*.[25] and Cavé *et al*.[18]

In conclusion, we describe two robust new dual-color split signal FISH assays for the detection of

t(5;14)(q35;q32) involving *HOX11L2* or *CSX*, or variant translocations involving these genes for use in both interphase and metaphase cytogenetics. Using these assays, we did not identify patients with a t(5;14)(q35;q32) involving *CSX*, indicating that the incidence of this alternative 5q35 translocation is probably low. However, we did identify 5 new cases with a t(5;14) involving *HOX11L2* with the 14q32 breakpoint centromeric to the *IGH* region. All five of our positive cases showed *HOX11L2* expression, as did 1 case without the t(5;14)(q35;q32), showing that *HOX11L2* expression may occur by non-cytogenetic means. Additionally, our results suggest that *TAL1* aberrations and the t(5;14)(q35;q32) translocation involving *HOX11L2* are highly unlikely to co-exist in one patient, thus confirming the idea that these aberrations reflect oncogenetically different T-ALL subgroups.

LJCMvZ: interpretation of data and primary responsibility for writing the paper; SCJMV: FISH analysis of patients, interpretation of data and revision of paper; ILMW-T: FISH analysis and RT-PCR of patients, interpretation of data and revision of paper; TSP and RAFM: probe development, cell lines and revision of paper; JJMvD, HBB and AWL: study design, interpretation of data and writing the paper. All authors approve the final version of the manuscript as it is submitted to Haematologica. The authors thank the cytogenetic technicians of the Tumour Cytogenetics Laboratory, Department of Clinical Genetics for the considerable effort they have put into preparing the metaphases and for their assistance in analyzing the karyotypes.

The authors reported no potential conflicts of interest.

Manuscript received December 22, 2003. Accepted April 4, 2004

References

1. Aplan PD, Raimondi SC, Kirsch IR. Disruption of the SCL gene by a t(1;3) translocation in a patient with T cell acute lymphoblastic leukemia. J Exp Med 1992; 176: 1303-10.
2. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science 1990; 250:1426-9.
3. Bernard O, Lecointe N, Jonveaux P, Souyri M, Mauchauffe M, Berger R, et al. Two site-specific deletions and t(1;14) translocation restricted to human T-cell acute leukemias disrupt the 5' part of the tal-1 gene. Oncogene 1991;6:1477-88.
4. Delabesse E, Bernard M, Landman-Parker J, Davi F, Leboeuf D, Varet B, et al. Simultaneous SIL-TAL1 RT-PCR detection of all tal(d) deletions and identification of novel tal(d) variants. Br J Haematol 1997;99:901-7.
5. Aplan PD, Lombardi DP, Kirsch IR. Structural characterization of SIL, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Mol Cell Biol 1991;11:5462-9.
6. Carroll AJ, Crist WM, Link MP, Amylon MD, Pullen DJ, Ragab AH, et al. The t(1;14)(p34;q11) is nonrandom and restricted to T-cell acute lymphoblastic leukemia: a Pediatric Oncology Group study. Blood 1990; 76: 1220-4.
7. Fitzgerald TJ, Neale GA, Raimondi SC, Goorha RM. c-tal, a helix-loop-helix protein, is juxtaposed to the T-cell receptor-beta chain gene by a reciprocal chromosomal translocation: t(1;7)(p32;q35). Blood 1991;78:2686-95.
8. Aplan PD, Johnson BE, Russell E, Chervinsky DS, Kirsch IR. Cloning and characterization of TCTA, a gene located at the site of a t(1;3) translocation. Cancer Res 1995;55:1917-21.
9. Francois S, Delabesse E, Baranger L, Dautel M, Foussard C, Boasson M, et al. Deregulated expression of the TAL1 gene by t(1;5)(p32;31) in patient with T-cell acute lymphoblastic leukemia. Genes Chromosomes Cancer 1998;23:36-43.
10. 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.
11. Helias C, Leymarie V, Entz-Werle N, Falkenrodt A, Eyer D, Costa JA, et al. Translocation t(5;14)(q35;q32) in three cases of childhood T cell acute lymphoblastic leukemia: a new recurring and cryptic abnormality. Leukemia 2002;16:7-12.
12. 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 TCRδ locus in acute lymphoblastic leukemias with t(5;14)(q34;q11). Leukemia 2002;16:2205-12.
13. 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.
14. MacLeod RA, Nagel S, Kaufmann M, Janssen JW, Drexler HG. Activation of HOX11L2 by juxtaposition with 3'-BCL11B in an acute lymphoblastic leukemia cell line (HPB-ALL) with t(5;14)(q35;q32.2). Genes Chromosomes Cancer 2003;37:84-91.
15. Mauvieux L, Leymarie V, Helias C, Perrusson N, Falkenrodt A, Lioure B, et al. High incidence of Hox11L2 expression in children with T-ALL. Leukemia 2002;16:2417-22.
16. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell 2002;1:75-87.
17. 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.
18. Cave H, Suciu S, Preudhomme C, Poppe B, Robert A, Uyttebroeck A, et al. Clinical significance of HOX11L2 expression linked to t(5;14)(q35;q32), of HOX11 expression, and of SIL-TAL fusion in childhood T-cell malignancies: results of EORTC studies 58881 and 58951. Blood 2004;103:442-50.
19. Drexler HG. The Leukemia-Lymphoma Cell Line FactsBook. London: Academic Press; 2000.
20. MacLeod RAF, Drexler H.G. Cytogenetic characterization of tumor cell lines. In: Langdon S, editor. Cancer Cell Culture Methods and Protocols. Totowa NY: Humana Press; 2003. p. 57-76.
21. van der Burg M, Smit B, Brinkhof B, Barendregt BH, Verschuren MC, Dib M, et al. A single split-signal FISH probe set allows detection of TAL1 translocations as well as SIL-TAL1 fusion genes in a single test. Leukemia 2002;16:755-61.
22. Joos S, Falk MH, Lichter P, Haluska FG, Henglein B, Lenoir GM, et al. Variable breakpoints in Burkitt lymphoma cells with chromosomal t(8;14) translocation separate c-myc and the IgH locus up to several hundred kb. Hum Mol Genet 1992;1:625-32.
23. Kuipers J, Vaandrager JW, Weghuis DO, Pearson PL, Scheres J, Lokhorst HM, et al. Fluorescence in situ hybridization analysis shows the frequent occurrence of 14q32.3 rearrangements with involvement of immunoglobulin switch regions in myeloma cell lines. Cancer Genet Cytogenet 1999; 109:99-107.
24. van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999;13:1901-28.
25. 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.
26. Wakabayashi Y, Watanabe H, Inoue J, Takeda N, Sakata J, Mishima Y, et al. Bcl11b is required for differentiation and survival of αβ T lymphocytes. Nat Immunol 2003; 4:533-9.