HOX11L2 expression defines a clinical subtype of pediatric T-ALL associated with poor prognosis

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The most frequent oncogenic activation events characterized in childhood T acute lymphoblastic leukemia (T-ALL) result in the transcriptional activation of genes coding for transcription factors. The main genes are *TAL1/SCL*, a member of the basic region helix-loop-helix gene family, and *HOX11L2*, a member of the homeobox-containing protein family. To gain insight into the pathogenesis of this type of hematologic malignancy, we analyzed 28 T-ALL samples. *SIL-TAL1*/SCL fusion was detected in 6 patients; expression of *HOX11L2* was observed in 6 patients and of *HOX11* in 3 patients. With one exception, these activations did not occur simultaneously in the same patients, and they allowed the subclassification of 50% of the patients. *SIL-TAL1* fusion was detected in association with *HOX11* expression in one patient and with a t(8;14)(q24;q11) in another. High expression of *LYL1*, *LMO2*, or *TAL1* was observed mainly in samples negative for *HOX11L2* expression. *HOX11L1* and *HOX11* expression were observed in one instance each, in the absence of detectable chromosomal abnormality of their respective loci, on chromosomes 2 and 10, respectively. *HOX11L2* expression was associated with a chromosome 5q abnormality, the location of the *HOX11L2* locus in each case tested. Finally, our data show that *HOX11L2* expression was a suitable marker for minimal residual disease follow-up and was significantly associated with relapse ($P = .02$). (Blood. 2002;100:991-997)

Introduction

Extensive characterization of chromosomal abnormalities specific for T-ALL led to the identification of several oncogenes whose expression was up-regulated under the influence of the transcriptional regulation elements of genes normally expressed during T-cell differentiation. These T-cell–specific oncogenes code for transcription factors, and their oncogenicity has been well demonstrated in mouse models.[1,2] However, though they have been proven useful for monitoring residual disease, these T-ALL–specific molecular abnormalities are present in only 20% to 30% of samples and do not allow improvement of risk-based therapies.[3,4]

The *HOX11* gene is an orphan homeobox gene isolated because of its transcriptional activation as a result of the t(10;14)(q24;q11) or t(7;10)(q35;q24) translocation of T-ALL.[5] We have recently characterized a T-ALL–specific cryptic chromosomal translocation, t(5;14)(q35;q32), which is present in approximately 20% of childhood T-ALL samples and is associated with strong expression of *HOX11L2*, a *HOX11*-related gene.[6,7] To gain insight into the relation between *HOX11L2* expression and the other known T-ALL abnormal molecular features, we analyzed a series of 28 children with T-ALL for the presence of other frequent oncogenic events. We analyzed those samples for the expression of *TAL1*/SCL, *LYL1*, *LMO2*[8,9] and the 3 *HOX11* family genes.[10] We also investigated for *SIL-TAL1* fusion and deletion of the *CDKN2A/CDKN2D* locus.[11] Relationships with immunologic and clinical data are presented.

Patients and methods

Patients

Patients were children with T-cell malignancies diagnosed at Trousseau Hospital (Paris, France) from March 1996 to September 2001. Diagnoses were based on standard morphologic and histochemical parameters of leukemic cells and on the expression the T-cell antigens, cytoplasmic CD3, CD2, CD5, CD7, and the absence of B-cell antigens. Thirty-two patients were given diagnoses of T-ALL during that time, and samples for 28 were available. Informed consent was obtained from patients and their parents according to the Declaration of Helsinki. Clinical data of T-ALL patients are summarized in Table 1. Samples from children with T-lymphoblastic lymphoma (5 patients), B-ALL (52 patients), and acute myeloblastic leukemia (AML, 15 patients) diagnosed during the same period of time were also used. Bone marrow (BM) or peripheral blood (PB) samples were obtained at the time of diagnosis and during cytologic remission or at relapse and were cryopreserved.

Patients were treated according to the French multicenter risk-adapted protocol FRALLE.[12] T-ALL patients were assigned to the high-risk arm. Corticoresistance—defined as the persistence of more than 1000 circulating blast cells per microliter after 8-day treatment (with 60 mg/m^2 prednisone per day and 1 intrathecal injection of methotrexate–cytosine–arabinoside–prednisolone) and/or M3 status[13]—defined as the persistence of more than 25% blasts in BM aspiration at day 21—were indications that patients should be moved to the very high-risk arm and should undergo allogeneic or

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Table 1. Clinical and biologic characteristics of 28 consecutive T-ALL patients

Characteristics	No.
Sex (female:male)	12:16
Median age, y (range)	6.6 (1-14)
WBC count, median (range)	104×10^9/L ($1-999 \times 10^9$/L)
Hemoglobin level, median (range)	8.7×10^9/L ($4.5-15 \times 10^9$/L)
Bulky disease*	14 of 28
With mediastinal enlargement	8 of 28
Without mediastinal enlargement	6 of 28
Bone marrow partial involvement†	1 of 28
Risk-adapted treatment arms	
High risk	18 of 27
Very high risk‡	9 of 27
Corticoresistant patients	11 of 27
M3 at day 21	5 of 28
Induction failure	2 of 28
2-year EFS rate	64.7%
Number of events	6 of 28
Overall survival rate	82.1%
Follow-up time, median	28 mo

*Defined by the presence of hepatomegaly or splenomegaly reaching the umbelic or lymphoadenopathy of more than 5 cm or mediastinal enlargement.

†One patient (UPNT26) had 67% blasts at diagnosis.

‡Patient UPNT17 is a 1-year-old child treated in INFANT protocol.

autologous bone marrow transplantation during the first complete remission (CR). Clinical data of the patients are presented in Table 1.

Immunophenotyping

Mononuclear cells isolated from bone marrow aspirates were stained with a standard panel of antibodies against CD2, CD5, CD7, CD3, CD1a, TCR αβ, TCR γδ, CD19, CD20, CD22, CD24, CD79α, IgM, CD10, CD34, HLA-DR, CD45, CD13, CD15, and CD33. Results were classified as positive when a given monoclonal antibody stained more than 20% of leukemic cells. At least 5000 cells per sample were analyzed using a FACSort cytometer and CELLQuest software (both from Becton Dickinson, Le Pont de Claix, France).

RNA and DNA methods

Total RNA was extracted from frozen patient samples using RNAble (Eurobio, Les Ulis, France) according to the manufacturer's instructions. RNA from normal fetal spleen, fetal thymus, and adult spleen were obtained from commercial sources. Adult thymus RNA was a kind gift from Karen Leroy (Hôpital Henri Mondor, Créteil, France), and 4 normal fetal thymi were a kind gift from Jelena Martinovic (Hôpital Necker–Enfants Malades, Paris, France). Normal peripheral blood lymphocyte RNA was extracted from healthy donor samples, and 5 normal bone marrow RNA samples were obtained from B-ALL patients in long-term remission (CR longer than 5 years). Genomic DNA was extracted using standard methods.

RNA was reverse transcribed from 1 μg total RNA in a final volume of 20 μL containing reverse transcription–polymerase chain reaction (RT-PCR) buffer (1 mM each dNTP, 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl pH 8.3), 10 U RNAsin (Promega, Madison, WI), 100 mM dithiothreitol, 100 U Superscript II (Gibco-BRL, Cergy Pontoise, France), and 25 μM random hexamers.

One hundred nanograms cDNA equivalent of RNA was analyzed in each PCR experiment. PCR was carried out in a final volume of 50 μL with 0.5 U AmpliGold polymerase (PE Applied Biosystems, Foster City, CA), 200 μM each dATP, dCTP, dGTP, 400 μM dUTP, 25 pmol each primer (see below), and 2.5 mM $MgCl_2$. Cycle parameters were set for 10 minutes at 95°C and for 15 seconds at 95°C, 40 seconds at 60°C, and 40 seconds at 72°C for 35 cycles. Specificity of each *HOX11*, *HOX11L1*, and *HOX11L2* RT-PCR product was confirmed by direct nucleotide sequence analysis.

Primers and probes

Screening for *HOX11L2*, *HOX11L1*, and *HOX11* expression was carried out by standard RT-PCR using the following primers: *HOX11L2* 2Fo, GCGCATCGGCCACCCCTACCAGA; *HOX11L2* 3Rw, CCGCTCCGCCTCCCGCTCCTC; *HOX11L1*-286Fo, AGCACCTGTGAGCGGGAGAAG; *HOX11L1*-413Rw, GYGCCTGGGCCCTCGGGTTTG; *HOX11*-712Fo, CTGGCCAAGGCGCTCAAAATG; and *HOX11*-810Rw, GGCCTCCCGTTCCTCCGCAGTC.

Primers and probe sequences used for real-time quantitative PCR amplification (RQ-PCR) of *HOX11L2* and *HOX11* were selected with the assistance of the computer program Primer Expres (PE Applied Biosystem). They were designed on different exons to avoid amplification from residual genomic RQ-*HOX11L2-3*, CAAGACCTGGTTCCAAAACCG; RQ-*HOX11* L2-4, AGGCTGGATGGAGTCGTTGA; probe, FAM-CAGCTGCAACACGACGCCTTCCAA-TAMRA; RQ-*HOX11*-1F, AAATGACCGATGCGCAGGT; RQ-*HOX11*-2R, GTTCGCTTGCTGCCTCTCG; and probe, FAM-AACCGGCGGACAAAGTGGAGACG-TAMRA. Expression analyses of *TAL1*, *LYL1*, and *LMO2* were performed using the primers described by Ferrando et al.[14]

The *CDKN2A/CDKN2D* copy number was estimated using an RQ-PCR assay starting from genomic DNA (adapted from[15]). *SIL-TAL1* fusion RNA was detected as recommended by the BIOMED consortium for minimal residual studies in acute leukemia.[16] Detection of the *ABL* transcript was performed as described.[17]

Real-time polymerase chain reaction

Theoretical and practical aspects of RQ-PCR have already been described.[18] Figure 1A shows changes in reporter fluorescence during PCR reactions starting from the known copy number (10^8 to 10^2) of the *HOX11L2* cDNA. Fluorescence was expressed as normalized F1/F2 against background fluorescence accumulated during the first 15 PCR cycles, whereas cycle number indicated the point of the reaction at which the fluorescence generated by the cleavage of the probe crossed a fixed threshold above the baseline (crossing point or Cp). The standard curve was obtained by correlation of the standard concentration versus the Cp value, as shown in Figure 1B. A strong correlation between the Cp and the *HOX11L2* copy number ($r > 0.99$) was found over a range of at least 6 orders of magnitude, with a PCR efficiency value of 90%. Similar results were obtained with *HOX11* standard and the endogenous control *ABL* (data not shown).

For each patient and control sample, the quality and quantity of RNA were assessed by the amplification of *ABL* gene transcripts in independent

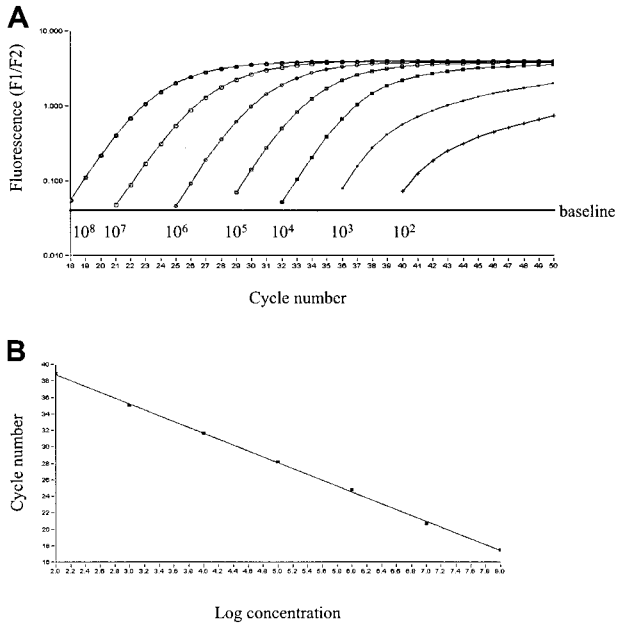

Figure 1. RQ-PCR analysis of *HOX11L2* expression. (A) Fluorescence data from *HOX11L2* standard dilution series. Curves show the relative fluorescence intensity with respect to the number of PCR cycles. Starting template copy numbers are indicated. (B) Linear relation between the cycle number and the logarithm of the initial template concentration. Mean slope value of the standard curve established in different series is −3.48, and the correlation coefficients ranged from 0.995 to 1.

RQ-PCR runs. Samples were considered eligible for testing only when the Cp of the internal reference *ABL* was lower than 30. Quantitative results were thus expressed as 1000 times the normalized copy number of the target gene against the copy number of the endogenous *ABL* gene.

PCR reactions were performed using the Light Cycler System (Hoffman-LaRoche, Grenoble, France). For each reaction, 100 ng reverse-transcribed RNA sample was added to 15 μL vol PCR mix containing 1× LC master mix, 5 mM $MgCl_2$, 300 μM each primer, and 200 μM probe. Thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 minutes followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Experiments were performed in duplicate for each data point. Each PCR run included the standard curve, a control without reverse transcriptase, and a control without template.

Cytogenetics and fluorescence in situ hybridization

Cytogenetic studies were performed in Hôpital Saint Antoine on bone marrow, blood cells, or both after short-term cultures for 17 and 24 hours. RHG banding technique was applied. Karyotypes are summarized in Table 2. Fluorescence in situ hybridization (FISH) was carried out using the usual techniques.[19] In addition to whole chromosome 5 and 14 painting probes (INSERM U301 and Appligene Oncor [Illkirch, France], respectively), chromosome 5 YAC clone (885a6) and BAC clones (already described or selected from available human sequence data bases) were used as probes as follows: *HOX11L2* (5q35)—593F7, 2248N14, 45L16; 14q32—2576L4, 1082A3; *CDKN2A/CDKN2D* (9p21)—145E5, 70L8; *HOX11* (10q24)—31L3; and *HOX11L1* (2p13)—140K4.

Statistical methods

Qualitative and categorized quantitative variables were compared to each other using χ^2 analysis with Yates correction. For event-free survival (EFS) time, the period taken into account was the interval between the diagnosis and an event or the last examination if no events occurred. Survival curves were calculated according to the Kaplan-Meier method with Statview 4.5 software (Abacus Concept, Berkeley, CA), and differences were assessed using the log-rank test.

Results

HOX11 gene family expression analysis

We used RT-PCR to detect *HOX11L2* expression in a panel of 28 pediatric patients with T-ALL, 5 with T-lymphoblastic lymphoma, 52 with B-ALL, and 15 with AML. No specific fragment could be amplified from the T-lymphoblastic lymphoma, B-ALL, or AML samples, whereas a single 244-nucleotide fragment was observed in 6 of 28 (21.4%) T-ALL samples (Figure 2; Table 2).

To evaluate the expression level of the *HOX11L2* gene, we developed a real-time quantitative RQ-PCR assay that was used to analyze the patients expressing *HOX11L2*. In these samples, the expression was homogeneously high, with *HOX11L2* normalized copy number ranging from 3984 to 21 680. In contrast, when

Table 2. Gene status, expression, and chromosome studies in 28 children with T-ALL

Patient	*HOX11L2*	*HOX11*	*LYL1*	*LMO2*	*TAL1*	*SIL-TAL1*	*CDKN2A/CDKN2D*	Karyotype
UPNT1	10 520	—	ND	ND	ND	−	ND	46,XY[11]
UPNT1 relapse	13 120	—	**112 000**	**64 366**	**136 084**	−	ND	ND
UPNT2	9 306	—	ND	ND	ND	−	−/−	46,XX[18]
UPNT3	—	—	**64 653**	**18 692**	**5 276**	−	±	46,XY,t(10;11)(p14;q14)[10]/92,idemx2,i(17)(q10)x2[13]/46,XY[1]
UPNT4	—	—	**23 603**	**20 407**	**47 845**	+	−/−	46,XY[12]
UPNT5	—	8	**11 360**	**19 775**	**14 852**	+	+/+	**Failure**
UPNT6	—	2 490	**27 637**	**14 068**	**34 184**	−	ND	Failure
UPNT7	—	—	**43 521**	**30 139**	3 500	−	+/+	45,XX,t(4;7)(p15-16;q11),-17[6]/46,XX[19]
UPNT8	8 580	—	**22 463**	1 329	**8 620**	−	−/−	46,XX,add(5q34)[3]/idem,del(9p21-22)[2]/46,XX[8]
UPNT8 relapse	11 549	—	ND	ND	ND	−	−/−	ND
UPNT9	—	—	3 581	743	**6 447**	+	−/−	47,XX,del(6q),t(8;14)(q24;q11),+ 20[18]/46,XX[8]
UPNT10	—	—	3 540	1 429	**8 675**	−	±	**Failure**
UPNT11	6 950	—	3 541	132	66	−	+/+	46,XY[18]
UPNT12	—	—	**26 103**	**19 299**	332	−	−/−	46,XY[14]
UPNT13	—	—	1 850	1 211	**23 115**	+	−/−	46,XX,add(13)(p13)[18]/46,XX[32]
UPNT14	—	—	**34 467**	**35 276**	**11 504**	−	ND	46,XX,add(1)(q?32),add(5)(p?13),-7,add(10)(p13-14),+ mar[25]/46,XX[5]
UPNT15	—	—	**22 308**	2 975	**4 659**	−	±	**Failure**
UPNT16	—	—	6 815	310	**66 377**	−	−/−	**Failure**
UPNT17	—	—	7 797	6 067	761	−	+/+	46,XX[24]
UPNT18	21 680	—	**13 326**	364	86	−	−/−	**Failure**
UPNT19	—	—	8 864	2 224	1 932	−	±	46,XY[26]
UPNT20	—	—	**13 590**	8 204	2 208	−	+/+	46,XY[22]
UPNT21	—	—	**59 218**	**13 397**	319	−	−/−	46,XY[17]
UPNT22	—	—	4 678	1 828	580	−	ND	46,XX[24]
UPNT23	—	—	ND	ND	ND	+	−/−	46,XY,del(9)(p21)[2]
UPNT24	3 984	—	7 895	2 251	717	−	−/−	46,XY[27]
UPNT25	—	—	1 333	1 022	1 124	−	−/−	46,XY,inv?del(2)[22]
UPNT26	—	—	**38 824**	**15 456**	**14 965**	−	±	Failure
UPNT27	—	34 388	**19 939**	321	78	−	−/−	46,XX,add(22)(q13)[4]/46,XX[28]
UPNT28	—	—	1 155	2 073	**3 956**	+	+/+	46,XY[26]
Thymus	—	—	250	191	436	—	—	—
Bone marrow	—	—	10 792	13 202	27 197	—	—	——

HOX11 and *HOX11L2* normalized copy numbers are indicated in positive samples.
+ indicates presence of the *SIL-TAL1* fusion or 1 copy of the *CDKN2A/CDKN2D* locus; −, absence of the *SIL-TAL1* fusion or 1 *CDKN2A/CDKN2D* locus; ND, not done.
Copy numbers of samples considered positive for *LYL1*, *TAL1*, and *LMO2* are in bold numbers.
Thymus and bone marrow expression level is the mean of 5 normal samples.

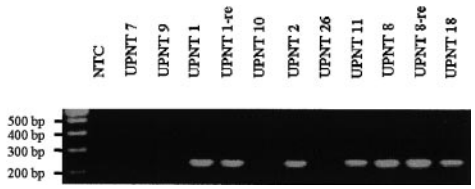

Figure 2. RT-PCR analysis of *HOX11L2* expression in T-ALL samples. A 242-bp fragment is amplified from several samples. NTC indicates no template control; re, relapse.

investigated in normal hematopoietic tissue, *HOX11L2* expression was not detected in adult spleen, adult peripheral blood lymphocytes, or control bone marrow. Using the same assay, *HOX11L2* was found to be expressed at a very low level in fetal thymus (1.2 normalized copy number), fetal spleen (0.57 normalized copy number), and adult thymus (0.7 normalized copy number). Taken together, these results are consistent with a restricted expression pattern of the *HOX11L2* gene and a specific association between high *HOX11L2* expression and T-ALL within leukemic samples.

The expression of *HOX11* was observed in 3 of 28 (10.7%) samples, though 1 (UNPT5) exhibited a very low level of expression when compared to the 2 others. Virtually no expression was detected in thymus or bone marrow controls. Results are summarized in Table 2. All patients except UNPT2 were searched for the expression of *HOX11L1*, the third member of the *HOX11* family. One (UPNT1) exhibited low expression of *HOX11L1* in addition to *HOX11L2* (data not shown).

Expression of other T-ALL oncogenes

We next searched for the expression of other T-cell oncogenes and for *TAL1*, *LYL1*, and *LMO2* genes. Because those genes are expressed during normal hematopoietic differentiation, we used RQ-PCR analyses to accurately estimate the level of gene expression. For *LYL1* and *LMO2*, samples expressing more than the bone marrow controls were considered positive.

For *TAL1*, samples expressing more than the weaker expressing *SIL-TAL1*–positive patient (UPNT28, see below) were considered positive. As shown in Table 2, using these thresholds, 14 patients were found positive for *LYL1*, 9 for *LMO2*, and 14 for *TAL1*. Of note, χ^2 analysis suggests a significant association between *LYL1* and *LMO2* expression ($P = .002$).

Oncogenic lesions

We next examined the T-ALL samples for the presence of other known T-ALL frequent, specific oncogenic events. The *SIL-TAL1* fusion is specific for T-ALL and is known to be due to an infra-microscopic (approximately 80-kb) deletion that leads to the transcription of the first exon of *SIL* fused to the coding sequences of *TAL1*. In our T-ALL patients, the *SIL-TAL1* fusion transcript was detected in 6 of 28 (21.4%) samples (Table 2), in keeping with previous reports.[8]

In one instance (UPNT5), *HOX11* expression was observed in a patient with *SIL-TAL1* fusion. No co-expression of *HOX11L2* with *SIL-TAL1* or *HOX11* was observed in any patient.

A frequent but nonspecific event observed in T-ALL is the inactivation of the *CDKN2A/CDKN2D* genes, occurring mainly through the deletion of 1 or 2 gene copies.[11] When genomic DNA was available, the number of *CDKN2A/CDKN2D* copy was estimated by RQ-PCR according to the described procedure.[15] The status of this locus was also investigated by FISH analysis using BAC clones specific for the *CDKN2A/CDKN2D* locus on 9 patients. Taken together, homozygous deletion of CDKN2A/CDKN2D copies was found in 13 of 24 samples (Table 2), in keeping with previous reports,[11] but does not appear to be obviously associated with the other specific lesions investigated here—*HOX11*, *HOX11L2*, and *SIL-TAL1* expression.

Cytogenetic and FISH studies

We next wanted to compare our molecular results with cytogenetic data. Karyotype analysis of the patient samples is summarized in Table 2. Two patients exhibited a recognized chromosomal translocation: UPNT3 exhibited t(10;11)(p14;q14), expected to result in a *CALM-AF10* fusion gene,[20] and UNPT9 showed t(8;14)(q24;q11), expected to lead to transcriptional activation of the c-*MYC* gene.[21] No cytogenetic abnormality of the *LYL1*(19p13), *TAL1* (1p32), or *LMO2* (11p13) loci was observed in these samples.

Because it detects an *SIL-TAL1* fusion transcript, the RT-PCR assay allows direct detection of the rearranged *TAL1* copy, which can be considered a bona fide oncogenic event. In contrast, because the known abnormalities of *HOX11* and *HOX11L2* genes result in transcriptional activation of these genes without any kind of fusion at the RNA level, RT-PCR analysis demonstrated only the expression of these genes, without any clue to the underlying molecular reasons. To establish whether the expression of these 2 genes was associated with a structural abnormality of the corresponding locus, we investigated the structure of chromosome 10, on which the *HOX11* gene lies, and of chromosome 5, on which the *HOX11L2* gene lies, using classical cytogenetic and FISH analyses.

Three samples show *HOX11* expression. No material for cytogenetic studies was available for UNPT5 or UPNT6. No 10q24 abnormality could be uncovered in UPNT27 through FISH using a specific BAC clone (data not shown). Based on our RQ-PCR data, *HOX11* was not detectably expressed during normal bone marrow differentiation, and the molecular reasons for its expression in patient samples without 10q24 abnormalities remain to be established. Similarly, no abnormality of the *HOX11L1* locus could be detected through FISH analysis using a specific BAC clone on UNPT1 material (data not shown).

HOX11L2 expression was observed in 6 patients, but material for cytogenetic studies was available for further investigation in only 4 patients. In 2 of them (UPNT11 and UPNT24), FISH analysis uncovered t(5;14)(q35;q32), known to be associated with *HOX11L2* expression. The involvement of the *HOX11L2* locus was further established using specific BAC probes (data not shown). In

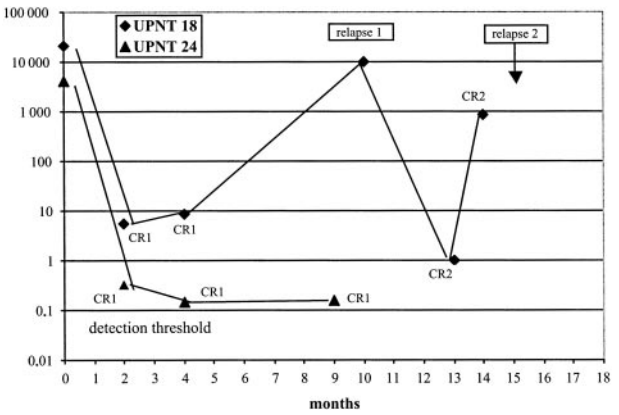

Figure 3. Minimal residual disease follow-up in 2 *HOX11L2*-expressing patients. Patient UPNT18 had a relapse 10 months after CR1. CR2 was achieved 4 months later. A second relapse occurred 16 months after diagnosis but could not be investigated. Patient UPNT18 remained in CR for 10 months after the beginning of treatment. Values are 8.8 and 10 *HOX11L2* normalized copy number for the 2 CR1 samples of UPNT18 and 0.3, 0.15, and 0.16 for the 3 CR1 samples of UPNT24.

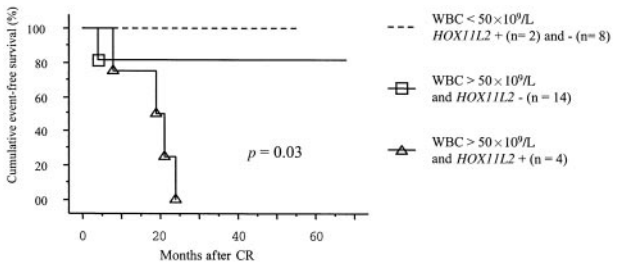

Figure 4. Event-free survival curves of patients stratified by WBC and expressing or not *HOX11L2*. Note that curves of patients expressing and not expressing *HOX11L2* are superimposed in the WBC $< 50 \times 10^9$/L group.

the 2 other patients, conventional karyotype was normal in UNPT1 and showed a der(5) in UNPT8 (Table 2). The involvement of the *HOX11L2* locus was assessed in these 2 patients using samples obtained in relapse through the use of chromosome 5 YAC and BAC probes. These probes gave a split signal on each patient's metaphase chromosomes, between a der(5) and a submetacentric chromosome identified by whole chromosome painting as chromosome 7 in each instance (data not shown).

HOX11L2 is a marker for minimal residual disease monitoring

Because *HOX11L2* expression is barely detected in normal hematopoietic tissues, we used RQ-PCR to monitor *HOX11L2* expression and to follow the disease course in 2 patients. *HOX11L2* level of expression was estimated in bone marrow samples from UPNT18 collected at complete remission (CR1) and 4 months after diagnosis. Samples were also analyzed at the time of patient UNPT18 relapse, which occurred 10 months after diagnosis, and were collected 13 and 14 months after diagnosis during CR2. A second relapse occurred 1 month after the last sample analyzed.

Similar analysis was performed on samples from UNPT24, collected at diagnosis and at CR1. Two samples, collected 4 and 9 months after diagnosis while the patient was still in CR1, were also investigated. As shown in Figure 3, samples from UPNT18 showed low but consistently detectable levels of *HOX11L2* expression, even in samples obtained after remission appeared as complete, based on cytologic and immunologic data. This high level of *HOX11L2* expression is likely to reflect the persistence of leukemic cells. The patient had a relapse 9 months after CR1, and again *HOX11L2* expression remained detectable in CR2. Furthermore, a dramatic increase of *HOX11L2* expression was observed 1 month before the occurrence of a second relapse. In contrast, in UPNT24 patient samples in CR1, *HOX11L2* expression rapidly decreased and remained at very low levels. These data illustrate the potential use of *HOX11L2* expression as a marker for minimal residual disease monitoring.

Relation between specific oncogenic events, immunophenotype, and clinical data

We next searched for an association between a specific immunophenotype and one of the T-ALL subgroup defined by the expression of *HOX11*, *HOX11L2*, or the presence of the *SIL-TAL1* fusion. Data are summarized in Table 3. As previously described, the *SIL-TAL1*–positive samples were not associated with a precise phenotype but are in keeping with a late cortical phenotype ($CD3^+$, $CD8^+$).

All the *HOX11L2*-positive samples were positive for CD1a and CD4; the association was close to significance with CD4 ($P = .055$). Most of the samples were also positive for CD10 (4 of 6; 66.6%), CD8 (4 of 6; 66.6%), and CD34 (4 of 6; 66.6%). When compared with *HOX11*-positive samples, the prominent differences are a less frequent expression of CD10 and CD34 in the latter samples. Taken together, these data indicate a slightly different immunophenotype for *HOX11*- and *HOX11L2*-expressing samples. Nevertheless, each reflected an early cortical stage of thymocyte differentiation.

Six patients in this series had relapses. Among them, 4 had been shown to express *HOX11L2* at diagnosis. Only one patient with *SIL-TAL1* fusion (and t(8;14)) and none with *HOX11* expression had relapses. Association of the clinical outcome (24-month EFS) with clinical and genetic data were submitted to statistical analysis. Selected results are shown in Table 4. WBC and *HOX11L2* expression were shown to be significantly associated with a relapse event ($P = .05$ and $P = .02$, respectively). Interestingly, as shown in Figure 4, when adjusted for WBC, *HOX11L2* expression remained significantly predictive of relapse in this series ($P = .03$).

Discussion

The high frequency of ectopic expression of the *HOX11L2* gene in childhood T-ALL prompted us to draw a broad molecular picture of this disease. We investigated a panel of 28 pediatric patients with T-ALL for the presence and association of the frequent known oncogenic activation, expression of the related *HOX11* and *HOX11L2* genes, *SIL-TAL1* fusion, and gross alteration of the *CDKN2A/CDKN2D* locus.

A surprisingly high number of samples was found to express at least 1 of 3 other T-cell oncogenes tested—*TAL1*, *LYL1*, and *LMO2*—in the absence of obvious abnormalities of their loci. In this series, the *LMO2*-positive samples also expressed *LYL1*, a feature of immature hematopoietic cells. *TAL1* expression in the

Table 3. Immunophenotype according to gene expression or genetic status of T-ALL samples

		CD34	CD10	CD2	CD1a	CD4	CD8	CD3	TCR αβ	TCR γδ	My
HOX11L2	+	4 of 6	4 of 6	5 of 6	5 of 5	6 of 6	4 of 6	3 of 5	2 of 5	1 of 5	3 of 6
HOX11L2	−	8 of 22	5 of 22	18 of 20	8 of 14	11 of 22	14 of 22	11 of 21	9 of 17	1 of 18	8 of 21
SIL-TAL1	+	1 of 6	1 of 6	6 of 6	3 of 5	3 of 6	6 of 6	5 of 6	4 of 5	0 of 5	3 of 5
SIL-TAL1	−	11 of 22	8 of 22	17 of 20	10 of 14	14 of 22	12 of 22	9 of 20	7 of 17	2 of 18	8 of 22
HOX11	+	0 of 3	1 of 3	2 of 2	2 of 2	3 of 3	3 of 3	1 of 2	1 of 2	0 of 2	0 of 2
HOX11	−	12 of 25	8 of 25	21 of 24	13 of 17	14 of 25	15 of 25	13 of 24	10 of 20	2 of 21	11 of 25
LYL1	+	6 of 14	5 of 14	8 of 14	5 of 7	9 of 14	7 of 14	3 of 12	3 of 10	1 of 10	7 of 12
LYL1	−	6 of 11	3 of 11	10 of 11	6 of 8	6 of 11	6 of 11	8 of 11	5 of 9	1 of 10	4 of 11
TAL1	+	6 of 13	4 of 13	11 of 12	5 of 7	7 of 13	10 of 13	8 of 11	7 of 9	0 of 8	5 of 11
TAL1	−	6 of 12	4 of 12	10 of 11	7 of 10	8 of 12	7 of 12	4 of 12	2 of 10	2 of 11	6 of 12
LMO2	+	3 of 9	3 of 9	6 of 7	2 of 4	5 of 9	6 of 9	3 of 8	3 of 7	0 of 7	5 of 8
LMO2	−	9 of 16	5 of 16	14 of 16	10 of 13	10 of 16	10 of 16	8 of 15	5 of 12	2 of 13	6 of 15

My indicates the presence of CD13, CD15, or CD33.

Table 4. Log-rank test of clinical and genetic data with patient outcome

Variable	Status (no. patients)	EFS rate at 24 mo [CI]	*P*
HOX11L2	+ (6)	20 [0.8-58]	.02
	− (22)	89 [64-97]	
SIL-TAL1	+ (6)	62 [29-83]	.75
	− (22)	80 [20-97]	
p16	Homozygous deletion (14)	56 [19-82]	.18
	Other (11)	80 [20-97]	
LYL1	+ (14)	74 [38-91]	.74
	− (10)	60 [8-90]	
LMO2	+ (9)	89 [43-98]	.14
	− (15)	42 [6-76]	
TAL1	+ (13)	80 [40-95]	.25
	− (12)	53 [8-85]	
WBC (10^9/L)	< 50 (10)	100	.05
	> 50 (18)	50 [20-74]	
Age (year)	< 9 (18)	65 [30-86]	.92
	> 9 (10)	71 [23-92]	
Sex	F (12)	70 [32-89]	.98
	M (16)	62 [20-87]	
CH R	− (23)	80 [20-97]	.75
	+ (5)	63 [39-83]	
CO R	− (16)	86 [33-98]	.15
	+ (11)	53 [19-79]	
CD1a	− (6)	80 [20-97]	.85
	+ (13)	60 [19-85]	
CD4	− (11)	90 [47-98]	.43
	+ (17)	60 [29-81]	
CD8	− (10)	67 [5-94]	.31
	+ (18)	62 [30-82]	
CD3	− (12)	89 [5-94]	.16
	+ (14)	56 [19-82]	
CD34	− (16)	74 [38-91]	.57
	+ (12)	54 [13-83]	

CI indicates confidence interval; CH R, chemical resistance; CO R, cortisone resistance.

absence of obvious genomic alteration of its locus has been reported in T-ALL samples, though the proportions are still controversial.[14,22-24] Whether expression of those genes reflects bona fide oncogenic activation of a transcriptional regulatory cascade or merely a block in differentiation remains to be established.

The expression of *HOX11* and *HOX11L2* and the presence of *SIL-TAL1* fusion was observed in 3 (10%), 6 (21%), and 6 (21%) patients, respectively, in keeping with previous reports.[2,8] As expected, a high frequency of *CDKN2A/CDKN2D* alterations was observed, that were not obviously associated with the other genetic features studied here. Similarly, *HOX11L2* expression was not associated with another known oncogenic activation. In contrast, *SIL-TAL1* fusion was observed in association with a c-*MYC* activation in one patient and with *HOX11* expression in another (UNPT5), indicating a possible collaboration between *TAL1*/SCL and these oncogenes in leukemogenesis.

However, *HOX11* was expressed at a very low level in UPNT5 in comparison with the 2 other patients expressing HOX11, challenging a direct role in leukemogenesis. In addition, HOX11 and HOX11L1 were expressed in 2 samples in the absence of any obvious chromosomal abnormality of the corresponding locus, a situation already reported for HOX11.[14,25] The mechanism and the meaning of these expressions are yet to be established.

HOX11L2 expression was detected in T-ALL but not in a series of B-ALL and AML patients, underscoring the specificity of the HOX11L2 ectopic expression. Cytogenetic and FISH analyses could be performed in 4 of 6 T-ALL patients expressing HOX11L2 and unraveled a t(5;14) translocation in 2 patients and a t(5;7) translocation in the 2 others. These data show that HOX11L2 expression in T-ALL is dependent on a molecular abnormality of 5q35. In addition, FISH analysis of 14 samples did not identify additional patients with a 5q35 abnormality (data not shown), confirming a tight relation between 5q35 abnormality and expression of HOX11L2. Because no or very low expression of HOX11L2 is detected in normal samples, we applied this quantitative assay to follow the outcome of 2 patients. Interestingly, the expression level of HOX11L2 was similar at diagnosis in both patients but differed markedly in the early times of treatment. One patient who maintained a relatively high level of HOX11L2 expression had a relapse, whereas the other patient showed a quick drop in the expression level of HOX11L2 and did not have a relapse for 10 months. These data suggest that monitoring HOX11L2 expression could be useful to follow the clearance of leukemic cells during the early phases of treatment, which is thought to be important for risk assessment.[26,27]

SIL-TAL1 fusion has not been associated with a distinct T-ALL clinical subgroup.[8] On the contrary, HOX11L2-expressing samples might define a T-ALL subgroup which shows a constant expression of CD1a and CD4 because all patients analyzed to date express both antigens.[6,7]

Successful treatment is achieved in approximately 60% to 75% of T-ALL patients. Interestingly, 4 of 6 patients in this series who had relapses were HOX11L2-expressing patients, whereas only one exhibited *SIL-TAL1* fusion and none expressed HOX11. All relapse events were observed in the group of patients with high WBC counts (greater than 50×10^9/L) at diagnosis. Interestingly, all patients of this group who expressed HOX11L2 had relapses. Although the small size of this series prevents any definitive conclusion, this observation indicates that HOX11L2 patients have poor prognoses. Additional studies are needed to validate these conclusions for clinical application.

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