IDENTIFICATION OF PROGNOSTIC GENETIC FACTORS IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

Identificatie van prognostische genetische factoren bij kinderen met T-cel acute lymfatische leukemie

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The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And behaving perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I–
I took the one less traveled by,
And that has made all the difference.

(Robert Frost, 1916)

Voor Marc, Vanessa
en mijn ouders

CONTENTS

I

General introduction

HEMATOPOIESIS AND LEUKEMIA

Hematopoiesis is a complex process in which primitive and undifferentiated stem cells multiply (self-renewal) and differentiate into many different blood cell types. Hematopoiesis primarily takes place in the bone marrow that is composed of stromal cells and a microvascular network, which forms a suitable microenvironment for stem cell growth and development. Hematopoiesis starts with a common pluripotent stem cell, which can give rise to myeloid or lymphoid progenitor cells. These progenitor cells further differentiate into various cell lineages. The myeloid progenitor cells can differentiate into red cells (erythrocytes), platelets (thrombocytes) and various white blood cells (leucocytes) (Figure 1).[1] Lymphoid progenitor cells develop into specific immunologic defence cells i.e. B-lymphocytes and T-lymphocytes.

Leukemia is a malignant transformation of hematopoietic progenitor cells. The accumulation of malignant blood cells in the bone marrow and peripheral blood results in a disturbed hematopoiesis, with anemia, bleeding, bruising and a decreased resistance to infections. Additional symptoms include fever, malaise, fatigue, abdominal organomegaly, enlarged lymph nodes, headaches, bone aches and weight loss. Depending on the cell lineage involved, a further distinction can be made in myeloid and lymphoid leukemias. According to the rate of malignant cell growth, leukemias are divided into acute and chronic leukemias. Eighty to eighty-five percent of the children with leukemia present with an acute lymphoblastic leukemia (ALL) whereas the remainder 15% has an acute myeloid leukemia (AML). Children with ALL can be further divided into ALL originating from the B-lineage ALL (precursor B-ALL) in 85-90% of the children and ALL originating from the T-lineage (T-ALL) in 10-15% of the children.

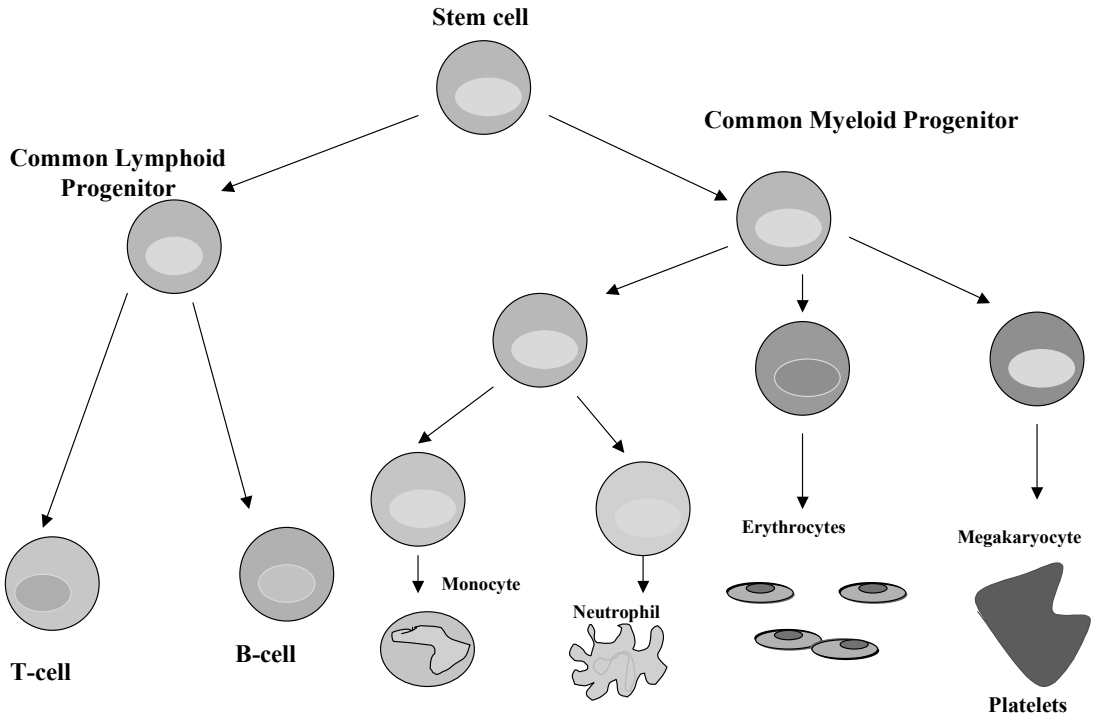

Figure 1. Normal Hematopoiesis, an overview
Adapted from Fischer et al., Nature Rev Immunol 2002, dec;2(12):977-82.[1]

Treatment of ALL

The treatment of ALL is based on combination chemotherapy that usually includes an induction phase, a consolidation phase, an intensification and a maintenance phase. Patients are stratified into different risk groups that receive different intensities of treatment based on their initial response to treatment and cytogenetics of the ALL cells. Unfavourable factors guide the patients towards more intensive treatment. The most important drugs used in the treatment of ALL are glucocorticoids (prednisolone and dexamethasone), vincristine, L-asparaginase, 6-mercaptopurine, methotrexate and antracyclines. During the last decade the prognosis of pediatric T-ALL has improved and the current 5 year relapse-free survival rate is 60-75%.[2-4] However, T-ALL still has a poorer treatment outcome compared to B-lineage ALL, which has a 5-year relapse-free survival of 80-85%.

Characterization of pediatric T-cell ALL

During normal T-cell development, early T-cells called thymocytes transiently home in the thymus, and differentiate into mature T-cells following several successive and distinctive developmental stages. The leukemic cells in T-ALL remain trapped into specific developmental stages with an increased cell growth potential. Characteristically, T-ALL is more frequent in males than females, presents with higher white blood cell count (WBC), has more frequent involvement of the central nervous system (CNS) than B-lineage ALL and is accompanied with an enlarged thymus at diagnosis.[5-8]

For B-lineage ALL patients, particular cytogenetic alterations including the *BCR-ABL* or *MLL* rearrangements are currently used for risk classification. Both alterations are strongly associated with poor prognosis and mandate the most intensive treatment, sometimes including allogenic stem cell transplantation. In T-ALL, the role of (cyto)genetic alterations and their clinical significance remains to be determined and is subject of this thesis. About 50% of T-ALL cases have been associated with specific molecular-genetic alterations or the expression of specific immunophenotypic markers, of which some have been related with survival rate.[9-11] Some chromosomal abnormalities occur in a mutually exclusive manner including abnormalities affecting the *TAL1* oncogene, translocations affecting the *LMO2* oncogene, *HOX11/TLX1* or *HOX11L2/TLX3* translocations. In some cases, translocations have been identified that give rise to fusion products such as *MLL*-fusion products, *BCR-ABL1*, or *CALM-AF10* fusion products. For about 40-50% of the T-ALL cases, such a unique abnormality has not been identified. Besides mutually exclusive abnormalities, recurrent abnormalities can be present irrespective the presence of aberrations as described above. These abnormalities include for example *p15/p16* deletions (in > 60% of the cases), *K-RAS* or *N-RAS* point mutations and *p53* inactivating mutations.

AIM OF THE THESIS

In this thesis, we aim to identify the prognostic relevance of recurrent T-ALL abnormalities that may delineate distinct molecular-cytogenetic entities in T-ALL. We

also investigate whether the presence of recurrent abnormalities are associated with arrest at distinct maturation stages, and whether this is related to outcome.

OUTLINE OF THE THESIS

Chapter 1. The aim of the study as described in this chapter was to define whether recurrent abnormalities in T-ALL affecting the *TAL1*, *HOX11* or *HOX11L2* oncogenes or that result in a *CALM-AF10* or *MLL* fusion product have prognostic relevance for pediatric T-ALL patients. Therefore the frequencies of these molecular-cytogenetic abnormalities in pediatric T-ALL patients were studied in the Dutch Childhood Oncology Group (DCOG) and the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL). Secondly, we investigated the relation of these cytogenetic alterations to clinical parameters, the expression of immunophenotypic markers and the expression of various oncogenic transcription factors including *TAL1*, *LYL1*, *LMO1* and *LMO2*.

Chapter 2. The aim of this chapter was to identify new and recurrent genetic abnormalities by means of the array-CGH technology. We report the identification of a new recurrent and cryptic deletion, i.e. the del(11)(p12p13) and how deletions affecting this chromosomal region can result in the activation of the *LMO2* oncogene. The relation of this deletion with other recurrent cytogenetic abnormalities, immunophenotypic characteristics as well as clinical outcome in pediatric T-ALL has been characterized.

Chapter 3. This chapter outlines the identification of new and recurrent abnormalities in T-ALL by clustering of gene expression signatures for the most recurrent and known T-ALL subgroups. We describe a new deletion in the 9q34 chromosomal region that results in the new and recurrent *SET-NUP214* fusion product. It was extensively investigated how this fusion product can activate the *HOXA* cluster of genes, as well as its effect on cell differentiation and proliferation.

Chapter 4. The aim of this study was to investigate a potential relationship between the presence of specific molecular-cytogenetic aberrations and arrest at specific T-cell developmental stages, and whether maturation arrest may determine the prognostic relevance for some of these chromosomal rearrangements. Maturation status was classified according to the European Group for the Immunological Characterization of Leukemias (EGIL) or the TCR classification system.

Chapter 5. CD34 is expressed by normal stem cells that highly express multidrug resistance genes making these cells relatively insensitive for chemotherapeutic compounds. CD34 expression in T-ALL may reflect an immature T-cell differentiation stage. Therefore, the expression of the *MDR1*, *MRP1* and *LRP* genes has been investigated in T-ALL patient samples and studied their association with CD34 expression as well as their prognostic significance.

Chapter 6. In this chapter we studied whether *ERBB2* is a suitable drug target in T-ALL. *ERBB2* expression appeared relatively high in *HOX11*-positive T-ALL compared to other subclasses of T-ALL. We investigated the sensitivity to the *ERBB2*-directed compound trastuzumab of a *HOX11*-positive T-ALL cell line in comparison to the sensitivity of *HOX11*-negative T-ALL cell lines.

Chapter 7. In this chapter, the current knowledge of molecular-cytogenetic abnormalities in T-ALL is reviewed that may represent distinct T-ALL subgroups, as well as their prognostic relevance. Moreover, the prognostic relevance of various pathways that have recently been identified in T-ALL, including the *NOTCH1* selfrenewal pathway as well as the *PTEN/AKT* survival pathway are discussed.

Finally, all results described in this thesis are summarized and discussed in the chapter "Summary".

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1

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

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ABSTRACT

Background and objectives
Subgroups of T-cell acute lymphoblastic leukemia (T-ALL), defined according to recurrent cytogenetic aberrations, may have different prognoses. The aim of this study was to determine the prognostic relevance of molecular-cytogenetic abnormalities in pediatric patients using quantitative real-time polymerase chain reaction and fluorescence *in situ* hybridization.

Design and methods
The patients were assigned to *TAL1, HOX11/TLX1, HOX11L2/TLX3,* or *CALM-AF10* subgroups. The cytogenetic subgroups were characterized in relation to immunophenotype and the expression of aberrantly expressed transcription factors.

Results
In our cohort study, *CALM-AF10* was associated with an immature immunophenotype and poor outcome (p=0.005). *HOX11L2* was associated with both immunophenotypically immature cases as well as cases committed to the γδ-lineage. *HOX11L2* was significantly associated with poor outcome (p=0.01), independently of the expression of CD1 or the presence of *NOTCH1* mutations. *TAL1* abnormalities were associated with αβ-lineage commitment, and tended to be associated with a good outcome. Cells in *HOX11* cases resembled early CD1-positive cortical thymocytes without expression of Cytβ, and TCR molecules. In relation to the expression of early T-cell transcription factors, high *TAL1* levels were found in immunophenotypically-advanced cases, whereas high *LYL1* levels were found in immature subgroups.

Interpretation and conclusions
The reported outcomes for *HOX11L2*-rearranged T-ALL cases are conflicting; the prognostic impact may depend on the therapy given. In our cohort, this cytogenetic aberration was associated with a poor outcome. Our data on *CALM-AF10* rearranged T-ALL, albeit based on only three patients, suggest that this type of leukemia is associated with a poor outcome.

INTRODUCTION

The outcome of pediatric acute lymphoblastic leukemia (ALL) has improved over the last decades leading to cure in approximately 80-85% of the cases. T-cell ALL, a malignant disease of thymocytes, accounts for about 10-15% of cases of pediatric ALL. T-ALL often presents with a high tumor load accompanied by rapid disease progression. About 30% of T-ALL cases relapse within the first 2 years following diagnosis.[1] About half of pediatric T-ALL patients have chromosomal translocations.[2,3] The most common chromosomal abnormalities include rearrangements affecting the T-cell receptor (TCR) genes.[4] These TCR genes are frequently translocated to basic helix-loop-helix (bHLH) genes (*MYC, TAL1, TAL2, LYL1, bHLHB1),* cysteine-rich (LIM-domain) genes (*LMO1, LMO2)* or homeodomain genes (*HOX11/TLX1, HOX11L2/ TLX3,* members of the *HOXA* cluster). Other translocations leading to the formation of specific fusion genes, such as *CALM-AF10,*[5] or *MLL* rearrangements have also been described.[2,3] Some recurrent chromosomal rearrangements of specific oncogenes have been associated with the expression of specific immunophenotypic markers reflecting T-cell developmental stages. For example, *TAL1*-rearranged cases are predominantly associated with a mature $CD3^+$, TCR α/β developmental stage.[6-8]

Specific T-ALL subgroups, defined according to immunophenotypic or cytogenetic aberrations, may have prognostic relevance. For instance, $CD1a^+$ T-ALL, representing a stage of intermediate or cortical thymic differentiation, is associated with a favorable outcome.[9,10] T-ALL cases carrying the t(10;14) leading to high expression of *HOX11* frequently express CD1,[11,12] and the outcome of this subtype is also considered favorable.[12-15] Expression profiling showed that this T-ALL subset highly expresses genes involved in cell growth and proliferation, possibly explaining the increased responsiveness of this subset to chemotherapeutic agents.[11,15] It also highly expresses the glucocorticoid receptor,[11] which might contribute to a high sensitivity towards dexamethasone.[16] High expression of *HOX11L2* has been related with a poor outcome in some studies,[11,17] but not in other studies.[12,18] High *HOX11L2* expression was even associated with good prognosis in one study.[19] t(10;11)(p13;q14-21) leading to the *CALM-AF10* fusion products is observed in 4-9% of T-ALL, and patients with this translocation may have a poor outcome.[5] The clinical relevance of *TAL1* rearrangements, such as the *SIL-TAL1* deletion or *TAL1* translocations (e.g. t(1;14)(p32;q24)), remains unclear, although a trend for a favorable outcome and low levels of minimal residual disease at the end of induction therapy have been described.[12,20,21]

In this study, we investigated the prognostic significance of various recurrent molecular-cytogenetic abnormalities in a pediatric cohort of patients with T-ALL from the Dutch Childhood Oncology Group (DCOG). Findings were validated in a second and independent T-ALL cohort of the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL). The cytogenetic subgroups were further characterized in relation to immunophenotype and the expression of aberrant transcription factors.

DESIGN AND METHODS

Patients

Viably frozen diagnostic bone marrow (n=41) or peripheral blood samples (n=31) from 72 pediatric T-ALL patients were randomly chosen from the DCOG collection of ALL tissue, thereby minimizing selection bias. The immunophenotypic data related to these samples were kindly provided by the DCOG. All patients were diagnosed between 1991 and 2000. Thirty patients (33%) were treated with the equivalent treatment protocols DCOG ALL-7[22] or ALL-8[23] (in total 34 and 56 T-ALL patients included, respectively) and 42 patients (47%) were treated according to the ALL-6[24]-equivalent ALL-9 protocol (in total 90 T-ALL patients included). Since the outcome for the ALL-7/-8 versus ALL-9 treated patients was not different, these DCOG patients are combined into one cohort in this study. All patient samples were processed as previously described and contained >90% of leukemic blasts.[25] A second cohort comprising 53 T-ALL patients treated with the COALL-97 protocol,[26] was used to validate the results. Informed consent was acquired and the study was approved by the ethical committee of the Erasmus Medical Center. Bone marrow biopsies (total 28 samples) obtained from ten children with no evidence of a hematologic malignancy, two children with Burkitt's lymphoma, five children with Hodgkin's disease and 11 children with solid tumors (5 neuroblastomas, 4 rhabdomyosarcomas and 2 Ewing sarcomas) were used to determine the reference levels of *TAL1, LYL1, LMO1* and *LMO2* expression.

RNA extraction and reverse transcription

RNA extraction and reverse transcription were performed according to a previously described procedure.[25]

Quantitative real-time reverse transcriptase polymerase chain reaction (RQ-PCR)

The expression levels of various target PCR as mentioned in the text were quantified relative to the expression level of the endogenous housekeeping gene, glycer-aldehyde-3-phosphate dehydrogenase (*GAPDH),* by real-time RT-PCR in an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) as described previously.[25,27] The *SIL-TAL1* primers (ENF601, ENR664) and probe (ENP641) for the detection of a *SIL-TAL1* deletion were used as described in the Europe Against Cancer Program.[28] Primers and probe for *GAPDH* have been described previously.[25] For *CALM-AF10,* 5' and 3' *CALM-AF10* fusion transcripts were detected in separate reactions using the *CALM-AF10* forward primer 5'-TTA ACT GGG GGA TCT AAC TG-3' in combination with the 5' fusion transcript reverse primer 5'-GCT GCT TTG CTT TCT CTT C-3 or the 3' fusion transcript reverse primer 5'-CCC TCT GAC CCT CTA GCT TC-3' in combination with the common *CALM-AF10* probe 5'-(FAM)-CTT GGA ATG CGG CAA CAA TG-(TAMRA)-3'. For detection of *HOX11* expression levels, the forward primer 5'-CTC ACT GGC CTC ACC TT-3' and reverse primer 5'-CTG TGC CAG GCT CTT CT-3' were used in combination with the probe 5'-(FAM)-CCT TCA CAC GCC TGC AGA TC-(TAMRA)-3'. For detection of *HOX11L2* expression levels, the forward primer

5'-TCT GCG AGC TGG AAA A-3' and reverse primer 5'-GAT GGA GTC GTT GAG GC-3' were used in combination with probe 5'-(FAM)-CCA AAA CCG GAG GAC CAA GT-(TAMRA)-3'. For the detection of *TAL1* transcripts, the forward primer 5'-TGC CTT CCC TAT GTT CAC-3' and reverse primer 5'-AAG ATA CGC CGC ACA AC-3' were used in combination with probe 5'-(FAM)-CCT TCC CCC TAT GAG ATG GAG A-(TAMRA)-3'. For detection of *LYL1* transcripts, the forward primer 5'-CGC TGC TGC AAC TCT C-3' and reverse primer 5'-ACC AGG AAG CCG ATG TA-5' were used in combination with probe 5'-(FAM)-CAC TTT GGC CCT GCA CTA CC-(TAMRA)-3'. For the detection of *LMO1* transcripts, the forward primer 5'-GCT GTA ACC GCA AGA TCA-3' and reverse primer 5'-GCT GCC CTT CCT CAT AGT-3' were used in combination with the probe 5'-(FAM)-CAA CGT GTA TCA CCT CGA CTG C-(TAMRA)-3'. For the detection of *LMO2* transcripts, the forward primer 5'-TTG GGG ACC GCT ACT T-3' and reverse primer 5'-ATG TCC TGT TCG CAC ACT-3' were used in combination with the probe 5'-(FAM)-AAG CTC TGC CGG AGA GAC TAT CT-3'.

Fluorescence in situ hybridization (FISH) analysis

Experiments were performed on interphase preparations as described in detail before.[29] Dual-colored FISH experiments to study the presence of a *CALM-AF10* fusion were performed using a combination of BAC-probes (BacPac Resources, Oakland, CA, USA) that are either localized centromeric (RP11-29E15) and telomeric (RP11-12D16) to the *CALM* breakpoints (split FISH), or telomeric to *CALM* (RP11-12D16) and centromeric to *AF10* breakpoints (RP11-399C16; fusion FISH). BAC were labeled with biotin-16-dUTP/digoxigenin-11-dUTP (Roche, Penzberg, Germany) by nick translation.[30] Samples were scored positive when more than 10% of interphase cells demonstrated a *CALM-AF10* fusion or *CALM* split apart signal for 100 counted cells by two independent observers. *MLL-, HOX11-, HOX11L2-* or *TAL1-* translocations or the *SIL-TAL1* deletion were determined by commercially available FISH kits provided by DakoCytomation (Glostrup, Denmark), and hybridized and scored as described by the manufacturer.

Immunophenotypic cytoplasmic TCRβ (Cytβ), TCRαβ and TCRγδ analyses

Indirect cytoplasmic TCRβ (Cytβ) staining was performed on acetone fixed cytospin preparations using the antibody βF1 (BioAdvance, France). Following incubation for 15 min with the unlabeled βF1 antibody, a secondary incubation with GαM-FITC was performed for visualization. Cytβ positivity was scored by two independent observers using fluorescence microscopy. TCR expression on the T-ALL samples was analyzed by standard flow cytometry using antibodies against TCRαβ (BMA031; Beckman Coulter, Fullerton CA, USA), and TCRγδ (11F2; Becton Dickinson, San José, CA, USA), in combination with staining for CD3 expression. Measurements and acquisition of data were done on FACScan and FACSCalibur flow cytometers (Becton Dickinson).

Statistical analysis

Kaplan Meier curves were constructed in SPSS 11.0 software, and *p*-values were determined using the log-rank test. The Cox model (p_{Cox}) was used to determine the independent prognostic importance of variables. An event was defined as having a

relapse or being a non-responder after induction therapy. Exploratory multivariate analyses of individual factors of potential prognostic significance (age, gender, white blood cell count) were performed using Cox-regression analysis. The Mann-Whitney U-test (MWU) was used to analyze differences in age, white blood cell count (WBC), and gene expression levels between subgroups.

RESULTS

Patients

The clinical characteristics of the DCOG cohort and specific subgroups are summarized in Table 1. In the first 2 years after initial therapy, 20/72 patients relapsed. No relationship was observed between outcome and age (> or ≤ 10 years; log-rank p=0.53), WBC (> or ≤50 x 10^9/L; log-rank p=0.31) or gender (log-rank p=0.19).

Recurrent chromosomal abnormalities in T-ALL reflect distinct T-ALL subgroups

Based upon FISH and RQ-PCR data, four distinct molecular cytogenetic subgroups could be identified, i.e. the *TAL1-, HOX11L2-, HOX11-,* and *CALM-AF10* rearranged subgroups and a subgroup designated as the *remaining-group* which included samples from patients lacking these abnormalities (Table 2). Fourteen patients (19%) had *TAL1* rearrangements: eleven had a *SIL-TAL1* deletion and three had a *TAL1* translocation. These patients were allocated to the *TAL1* subgroup. Patients with a *SIL-TAL1* deletion were positive using both RQ-PCR and FISH. None of these *SIL-TAL1* deleted patients had evidence of chromosome 1 abnormalities upon karyotypic analysis. The three patients with a *TAL1* translocation were negative for *SIL-TAL1* RQ-PCR as expected. FISH data for these three patients were in agreement with karyotypic data, with two patients having t(1;14)(p32;q11) and the other having the alternative t(1;7)(p31;q32).

Seventeen patients (24%) strongly expressed the *HOX11L2* homeobox gene, and are referred to as the *HOX11L2* subgroup. In 16/17 patients, *HOX11L2* rearrangements could be validated using FISH (Table 2). One patient highly expressed *HOX11L2* but lacked a FISH-detectable translocation, possibly due to a translocation variant not detected by our FISH procedure. FISH hybridization patterns suggested the presence of variant *HOX11L2* rearrangements[31] in four T-ALL patients to be described elsewhere (*manuscript in preparation).* None of the patients in the *HOX11L2* subgroup had been identified as having the t(5;14)(q35;q32) or other *HOX11L2* translocation variants by conventional karyotyping. One additional patient was detected with a *HOX11L2* translocation in 20% of the cells lacking detectable *HOX11L2* expression by RQ-PCR. This patient was not included in the *HOX11L2* subgroup, and is described below in further detail. Six cases (8%) expressed the homeobox gene *HOX11,* as determined by RQ-PCR. FISH showed translocations involving *HOX11* in all six cases (*HOX11* subgroup). Conventional karyotyping had demonstrated the presence of 10q24 rearrangements in only half of the patients: the t(10;14)(q24;q11) in two cases and the t(7;10)(q35;q24) in one case.

Table 1. Clinical characteristics of pediatric T-ALL cohorts according to molecular cytogenetic subgroups

	DCOG cohort	*TAL1*	*CALM-AF10*	*HOX11L2*	*HOX11*	Remaining*	COALL cohort	*TAL1*	*HOX11L2*	Remaing**
Total, n	72	14	3	17	6	32	53	11	7	35
Sex, n (%)										
Male	51	11 (78.6)	3 (100)	13 (76.5)	5 (83.3)	19 (59.4)	32	9 (81.8)	6 (85.7)	17(48.6)
Female	21	3 (21.4)	0 (0)	4 (23.5)	1 (16.7)	13 (40.6)	21	2 (18.2)	1 (14.3)	18 (51.4)
Age at diagnosis, years										
Median	6.8	10.1	10.1	5.9	8.9	5.8	7.8	4.7	8.4	10
Range	1.1-16.7	3.3-15.4	7.9-10.8	3.2-12.3	3.5-11.2	1.1-16.7	1.7-17.8	2.3-9.9	5.8-11.9	1.7-17.8
WBC count, 10 x ^{9}/L										
Median	135	160.5	124.1	98	102.8	136	110	118	81.1	110
Range	5.3-900	27.6-590	13.7-347	31.8-500	27.2-280	5.3-900	1.8-490.7	30-450	1.8-350	8.5-490.7

Remaining; Remaining group of the DCOG cohort: patients negative for TAL1, HOX11L2, HOX11 or CALM-AF10 rearrangements; Remaining**; Remaining group of the COALL cohort: patients negative for TAL1 and HOX11L2 rearrangements.*

Table 2. Characteristics of molecular cytogenetic aberrations in DCOG T-ALL patients

Pat. nr	M/F	Age (yrs)	WBC x10^9/l	Protocol	*SIL-TAL* RT-PCR	*HOX11L2* FISH	*SIL-TAL* FISH	*HOX11L2* RT-PCR	*HOX11* RT-PCR	*HOX11* FISH	*CA 3'* RT-PCR	*CA 5'* RT-PCR	*CA* FISH	*MLL* FISH	Karyotype	Relapse CCR	FU DFS	Status
1	M	7,5	130	ALL9	0,005	wt	Del	0	0	wt	0	0	wt	wt	46,XY[32]	CCR	44	alive
2	F	8	132	ALL9	0,014	wt	Del	0	0	wt	0	0	wt	wt	46,XX,?add(9)(p1?)[6]/46,XX[30]	CCR	35	alive
3	M	11,9	110	ALL7	0,022	wt	Del	0	0	wt	0	0	wt	wt	46,XY,del(6)(q1?q2?),del(9)(p?)[19]/ 46,XY,t(1,19)(q23,p13),del(9)(p1?)[3]	CCR	138	alive
4	M	14,9	300	ALL8	0,062	ND	ND	0	0	ND	0	0	wt	ND	46,XY[19]	CCR	139	alive
5	M	4,3	205	ALL8	0,022	ND	Del	0	0	ND	0	0	wt	ND	45,XY,del(6)(q15q25),dic(9;13)(p11;q13)[34]	CCR	105	alive
6	M	15,4	200	ALL8	0	wt	Trans	0	0	wt	0	0	wt	wt	46,XY,t(1;14)(p32;q11)/47,XY,t(1;14)(p32;q11),+mar	R	14	alive
7	M	11,3	192	ALL8	0	wt	Trans	0	0	wt	0	0	wt	wt	46,XY,t(1;7)(p31;q32)(52%)/46,XY (48%)	CCR	87	alive
8	F	9,8	27,6	ALL9	0,018	wt	Del	0	0	wt	0	0	wt	wt	45,XX,der(9)t(9;14)(p13;q1?2),del(12)(p12p13),-14,idic(17)(p11)[38]	CCR	61	alive
9	M	3,3	590	ALL9	0,011	wt	Del	0	0	wt	0	0	wt	wt	46,XY[30]	CCR	56	alive
10	M	10,4	112,2	ALL9	0,045	wt	Del	0	0	wt	0	0	wt	wt	46,XY,dup(17)(q12q24)[14]	CCR	41	alive
11	M	13,4	41,4	ALL9	0,090	wt	Del	0	0	wt	0	0	wt	wt	45,XY,-14[13]/46,XY[16]	CCR	31	alive
12	M	9,3	310	ALL9	0	wt	Trans	0	0	wt	0	0	wt	wt	46,XY,t(1;14)(p32;q11)inc[2]	CCR	75	alive
13	F	13	95	ALL9	0,049	wt	Del	0	0	wt	0	0	wt	wt	46,XX[28]	CCR	2	td
14	M	6,4	188,1	ALL9	0,008	wt	Del	0	0	wt	0	0	wt	wt	46,XY[30]	CCR	54	alive
15	M	5,5	185	ALL9	0	Trans	wt	0,27	0	wt	0	0	wt	wt	46,XY,ins(5)(q3p1?4p1?2)[15]/46,XY[4]	CCR	32	alive
16	M	3,2	417	ALL9	0	Trans	wt	0,40	0	wt	0	0	wt	wt	46,XY,t(7;9)(p1?3;p2?2)[30]/46,XY[4]	CCR	33	alive
17	M	5,9	276	ALL9	0	Trans	wt	1,50	0	wt	0	0	wt	wt	46,XY,del(11)(q2?1q2?4)[21]/46,XY,add(9)(q11),del(11)(q2?1q2?4)[4]	R	11	dod
18	M	4,5	405	ALL8	0	Trans	wt	1,84	0	wt	0	0	wt	wt	46,XY	R	12	dod
19	F	7,8	89,5	ALL8	0	Trans *	ND	1,19	0	ND	0	0	wt	ND	46,XX[20]	CCR	122	alive
20	M	5,8	500	ALL7	0	Trans	ND	1,39	0	ND	0	0	wt	ND	UNKN	CCR	50	alive
21	M	5	140	ALL8	0	Trans	wt	1,43	0	wt	0	0	wt	wt	46,XY(100%)	R	12	dod
22	M	7,2	354	ALL8	0	Trans *	ND	17,56	0	ND	0	0	wt	ND	46,XY[6]	CCR	61	alive
23	M	6,3	35,9	ALL9	0	Trans *	wt	0,87	0	wt	0	0	wt	wt	46,XY,add(5)(q3?5),-14,+mar[17]	CCR	55	alive
24	M	6,2	68,9	ALL8	0	Trans *	wt	8,22	0	wt	0	0	wt	wt	46,XY[10]	R	11	dod
25	F	6,4	98	ALL8	0	wt	wt	0,39	0	wt	0	0	wt	wt	45,XX,dic(7;8)(p12;p12)[7]	R	8	dod
26	F	12,3	34,1	ALL9	0	Trans	wt	0,28	0	wt	0	0	wt	wt	46,XX,del(9)(p2?1)[9]/46,XX,add(9)(p12)[6]	CCR	11	td
27	M	6,3	174	ALL9	0	Trans	wt	1,23	0	wt	0	0	wt	wt	46,XY,t(1;7)(p34;q34)[15]	R	9	dod
28	M	5,1	80	ALL9	0	Trans	wt	0,51	0	wt	0	0	wt	wt	46,XY[30]	CCR	40	alive
29	M	5,3	31,8	ALL9	0	Trans	wt	0,27	0	wt	0	0	wt	wt	46,XY[32]	CCR	41	alive
30	F	8,8	88,5	ALL8	0	Trans	wt	0,27	0	wt	0	0	wt	wt	46,XX(100%)	CCR	75	alive
31	M	5,1	44,9	ALL8	0	Trans	wt	1,69	0	wt	0	0	wt	wt	47,XY,+8[4]	R	17	dod
32	M	10,3	149	ALL9	0	wt	wt	0	0,017	Trans	0	0	wt	wt	46,XY,t(10;14)(q2?4;q11)[2]/46,idem,del(12)(p11)[10]/46,idem,t(6;7)(p21;q34~35),del(12)(p11)[3]/46,XY[9]	CCR	36	alive
33	F	4,4	280	ALL7	0	wt	wt	0	0,048	Trans	0	0	wt	wt	46,XX	CCR	9	td
34	M	7,8	159	ALL8	0	wt	wt	0	0,189	Trans	0	0	wt	wt	46,XY,t(7;10)(q36;q25-q26)	CCR	80	alive
35	M	11,2	53,9	ALL9	0	wt	wt	0	0,059	Trans	0	0	wt	wt	46,XY,t(10;14)(q24;q11)[24]	R	37	alive
36	M	9,9	27,2	ALL8	0	wt	wt	0	0,334	Trans	0	0	wt	wt	46,XY[32]	CCR	95	alive
37	M	3,5	56,6	ALL9	0	wt	wt	0	0,237	Trans	0	0	wt	wt	47,XY,+del(9)(p1?1)[11]	CCR	62	alive
38	M	10,8	347	ALL9	0	Trans *	wt	0	0	wt	0,013	0	Trans	wt	46,XY,t(10;11)(p13;q21)[20]	R	22	dod
39	M	10,1	13,7	ALL9	0	wt	wt	0	0	wt	0	0,0021	Trans	wt	46,XY,t(2;9)(q21;q34),?t(8;8)(?q22;q?24)[18]/46,XY[6]	R	19	dod
40	M	7,9	124,1	ALL9	0	wt	wt	0	0	wt	0,019	0	Trans	wt	46,XY,del(9)(p13p22),t(10;11)(p13;q21)[30]	R	9	dod
41	F	2,2	153	ALL9	0	wt	wt	0	0	wt	0	0	wt	Trans	46,XX,t(6;11)(q27;q23)[10]/47,XX,t(6;11)(q27;q23),+8[6]/46,XX[8]	CCR	36	alive
42	F	12	41,3	ALL9	0	wt	wt	0	0	wt	0	0	wt	Trans	48,XX,t(6;11)(q27;q23),+i(9)(q10),del(12)(p11),+21	CCR	36	alive
43	M	1,5	135	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,del(6)(q1?4q2?2),t(6;7)(q24;q35)[16]/46,XY	CCR	26	alive
44	F	9,3	900	ALL8	0	wt	NE	0	0	wt	0	0	wt	wt	46,XX,del(9)(p13p23)/46,XX,del(9),del(13)(q14q22)/46,XX,del(6)(q13q23),del(9)/46,XX,del(6),del(9),del(13)	R	16	dod
45	F	16,7	86,3	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XX,del(6)(q13q21),t(8;14)(q24;q11),del(9)(p22)	CCR	22	td
46	M	15,7	128,7	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,del(6)(q15q21),del(14)(q11q32),der(14)inv(14)/46,idem,dup(1)?(q21q42)[2]/46,idem,del(17)(p11)[4]	CCR	31	alive

47	M	15,9	136	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,del(6)(q2?q2?)[17]	R	8	dod
48	F	2,8	57,6	ALL8	0	wt	wt	0	0	wt	0	0	wt	wt	46,XX,del(6)(q?)[8]	CCR	119	alive
49	M	13,9	600	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY[26]	R	14	dod
50	F	8,4	15,4	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	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]	CCR	33	alive
51	F	3,8	158	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XX[28]	CCR	31	alive
52	M	13,7	UNKN	ALL8	0	ND	ND	0	0	ND	0	0	wt	ND	46,XY,?der(9)(p),add(12)(p12)[21]	CCR	109	alive
53	M	8,5	130	ALL8	0	ND	ND	0	0	ND	0	0	wt	ND	47,XY,+8	CCR	116	alive
54	F	4,3	5,3	ALL8	0	ND	ND	0	0	ND	0	0	wt	ND	46,XX,del(14)(q21q31)[4]	CCR	98	alive
55	M	6,2	167,2	ALL8	0	wt	wt	0	0	wt	0	0	wt	wt	47,XY,+?6[6]	R	7	dod
56	M	2,8	649	ALL7	0	ND	ND	0	0	ND	0	0	wt	ND	UNKN	CCR	18	alive
57	M	3,8	91,5	ALL8	0	wt	NE	0	0	wt	0	0	wt	wt	46,XY,t(14;20)(q32;q12)[2]/46,idem,i(9)(q10)[13]/46,idem,del(6)(q13q23),i(9)(q10)[2]/ 45,idem,-9,i(9)(q10)[2]/87-91,idemx2,-4,-8,-9,i(9)(q10)o	CCR	89	alive
58	F	1,9	387	ALL8	0	ND	ND	0	0	ND	0	0	wt	ND	46,XX[30]	CCR	96	alive
59	M	9,1	226	ALL8	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,t(11;14)(p15;q11)[3]	CCR	82	alive
60	M	1,6	50	ALL8	0	wt	wt	0	0	wt	0	0	wt	wt	UNKN	CCR	87	alive
61	M	6,2	191	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY[30]	CCR	39	alive
62	M	3,9	435	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY[30]	CCR	18	td
63	F	4,7	212	ALL8	0	wt	wt	0	0	wt	0	0	wt	wt	46,XX[32]	R	9	dod
64	F	15,9	231	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,X,del(X)(p21),del(5)(q31?),i(9)(q10),add(10)(p1?)	CCR	66	alive
65	F	13,2	49,5	ALL8	0	wt	wt	0	0	wt	0	0	wt	wt	46,XX	CCR	73	alive
66	M	9	95,2	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,t(11;14)(p13;q11)[10]	R	18	dod
67	M	1,1	529,5	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY[31]	CCR	44	alive
68	M	1,8	250	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,t(X;11)(q2?4;p13),add(3)(p24)[26]	CCR	50	alive
69	M	2,3	124,5	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY[20]	CCR	51	alive
70	M	13,8	128,9	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY,t(8;14)(q24;q11)[14]	R	13	dod
71	M	1,3	177	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	46,XY[31]	R	8	dod
72	F	5,3	60	ALL9	0	wt	wt	0	0	wt	0	0	wt	wt	47~48,XX,add(1)(p36)[4],+add(1)(p36)[3],-5[2],del(6)(q?)[2],-18[2],+mar[2][cp4]	CCR	63	alive

M, male; F, female; Age, age in years at diagnosis; WBC, white blood cell count; ALL7/8/9, patients treated according to DCOG protocol ALL7, ALL8 or ALL9; FISH results are scored as wt, wild type; Del, SIL-TAL1 deletion; Trans, translocation; Trans, variant translocation; ND, not done; NE, not evaluable; CA 3', CALM-AF10 3' fusion transcript; CA 5', CALM-AF10 5' fusion transcript; R, relapse; CCR: continuous complete remission; FU DFS: follow-up disease-free survival (in months); td, toxic death; dod: died of disease.*

Three cases (4%) expressing 5' and/or 3' *CALM-AF10* fusion transcripts, indicative of the presence of the t(10;11)(p13;q14-21), were identified using RQ-PCR. These patients were positive in the *CALM* split FISH and *CALM-AF10* fusion FISH procedures. FISH analysis of one of the *CALM-AF10* positive samples (from patient #38) demonstrated the presence of this translocation in about 80% of the leukemic cells, while the remaining 20% had a *HOX11L2* translocation possibly reflecting a *HOX11L2*-positive leukemic subclone. Since no evidence was found for *HOX11L2* gene expression in this patient, this patient was included in the *CALM-AF10* subgroup instead of the *HOX11L2* subgroup. Analysis of the relapse sample of this same patient using FISH revealed that 93% of the blasts had a *CALM-AF10* rearrangement indicating that this patient had been correctly allocated to the *CALM-AF10* subgroup. All other patients were included in a *remaining*-group. This subgroup included two *MLL*-rearranged cases (patients #41 and #42, both having a t(6;11)(q27;q23) by conventional karyotyping involving *MLL-AP6),* two cases with t(8;14)(q24;q11) (patients #45 and #70), one patient with a t(11;14)(p15;q11) (patient #59), one patient with a t(7;11)(q35;p15) (patient #55) and one patient with a t(11;14)(p13;q11) (patient #66), possibly involving rearrangements of *c-MYC, LMO-1,* or *LMO-2,* respectively (Table 2).

Outcome of patients in the different T-ALL subgroups

The clinical characteristics of each subgroup are indicated in Tables 1 and 2. For each subgroup, the relationship with outcome was assessed by Kaplan-Meier curves (Figure 1 and Table 3A).

The *TAL1* subgroup had the highest median age at diagnosis (10.1 years compared to 6.2 years for the *TAL1*-negative patients: MWU, p=0.022). The *TAL-1* subgroup had a trend towards a better outcome with a 3-year disease-free survival of 92±7.4% compared to 68±6.2% for non-*TAL1* patients (Figure 1A, log-rank p=0.0715). So far, only one out of 14 *TAL1*-rearranged patients has relapsed. Given that about half of the samples of the *remaining*-group, as well as single T-ALL samples characterized by the *HOX11*- or *HOX11L2*-rearrangements expressed levels of *TAL1* equal to those in *TAL1*-rearranged cases, it was investigated whether high *TAL1* levels (Figure 2A) and those expressing lower *TAL1* levels had similar 3-year disease-free survival rates (72±7% vs 72.4±8.3%; log-rank p=0.87). The 3-year disease-free survival rate of patients with *HOX11L2*-rearrangements was lower than that of the patients without *HOX11L2* rearrangements (57%±12.3% vs 77±5.89%; log-rank p=0.117; Figure 1B), although this difference was not statistically significant. The presence of *CALM-AF10* rearrangements was significantly associated with poor outcome (Figure 1C): all three patients relapsed 9, 19 and 22 months after diagnosis and subsequently died. No difference in outcome was observed for the *HOX11*-rearranged subgroup compared to the remainder of patients. One *HOX11*-positive patient relapsed more than 3 years after the start of therapy. We also tested potential confounding effects of other parameters including age, gender and WBC on outcome in multivariate analyses (Table 3B). The *CALM-AF10* rearrangement remained an independent predictive variable (p=0.04). Compared to the results of univariate analyses, stronger trends towards an association with good or poor outcome were

found for the *TAL1* (p=0.056) and *HOX11L2* subgroups (p=0.078). To validate the relation of *TAL1* and *HOX11L2* abnormalities and outcome, a second cohort consisting of 53 pediatric T-ALL patients enrolled in the German COALL-97 protocol was investigated. One patient was found to express both *CALM-AF10* 3' and 5' fusion transcripts, and was confirmed to have *CALM-AF10* translocation using FISH. The clinical characteristics are summarized in Table 1. The disease-free survival of the COALL patients did not differ from that of the DCOG patients. Male gender in the COALL cohort was associated with poor outcome with a 3-year disease-free survival of 59±9.1% vs 89±7.0% for female patients (log-rank p=0.013). We therefore decided to analyze this cohort separately from the DCOG T-ALL cohort. The outcome for COALL patients was independent of age or WBC. *TAL1* rearrangements in the COALL cohort were not associated with an improved outcome. *HOX11L2* abnormalities were significantly associated with a poor outcome (Figure 1D, log-rank p=0.014). *HOX11L2* rearrangements remained predictive for poor outcome in multivariate analysis (p=0.039). Also in an overall stratified analysis of the combined DCOG and COALL cohorts (n=125), both *CALM-AF10* (log-rank p=0.047) and *HOX11L2* (log-rank p=0.01) were predictive of a poor prognosis.

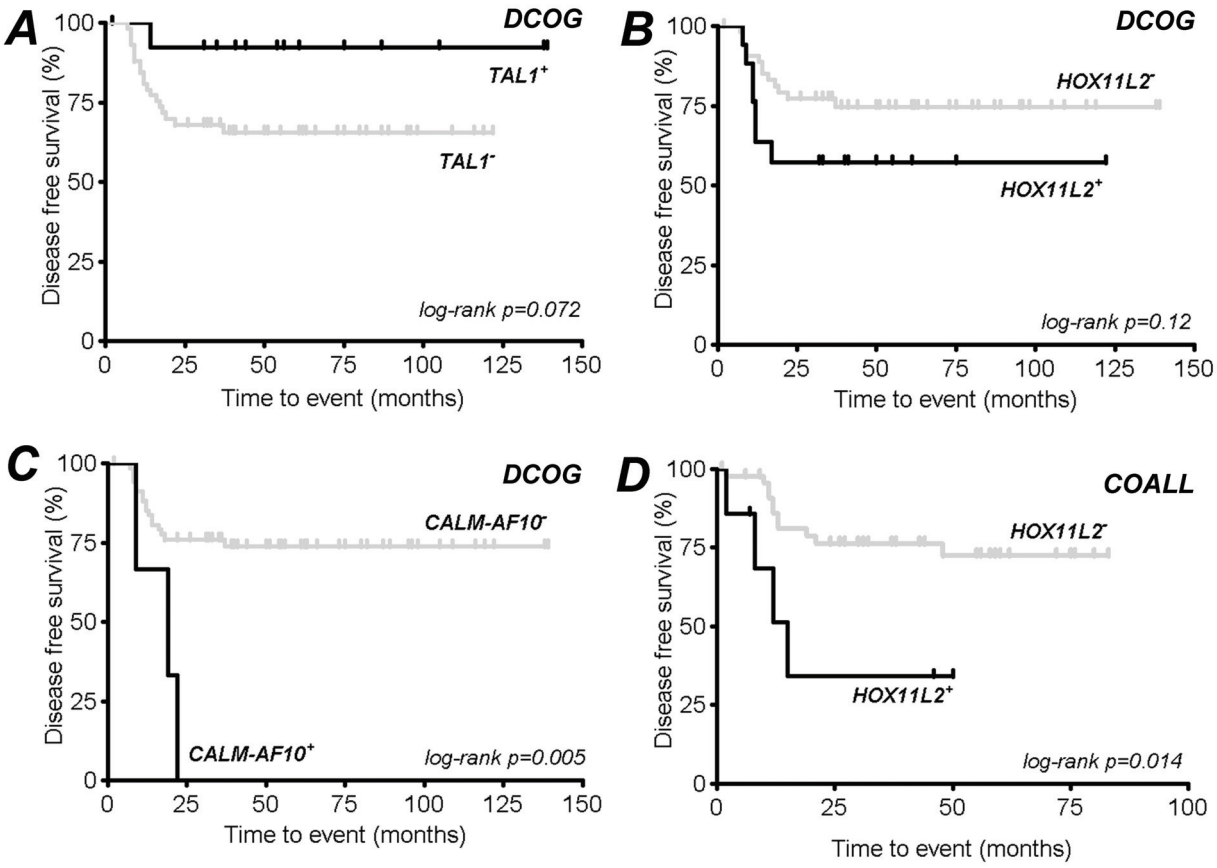

Figure 1. Disease-free survival of pediatric T-ALL patients from the DCOG cohort (n=72)
Disease-free survival for (A) the TAL1 rearranged (TAL1$^+$) versus non-TAL1 rearranged (TAL1$^-$), for (B) HOX11L2 rearranged (HOX11L2$^+$) versus non-HOX11L2 rearranged (HOX11L2$^-$); (C) CALM-AF10 rearranged (CALM-AF10$^+$) versus non-CALM-AF10 rearranged (CALM-AF10$^-$) T-ALL subgroups. (D) Disease-free survival for HOX11L2 rearranged (HOX11L2$^+$) versus non-HOX11L2 rearranged (HOX11L2$^-$) T-ALL subgroups from the COALL cohort (n=53).

Table 3. Uni- and multivariate analyses of the DCOG T-ALL patient samples

	A. Univariate Analyses using Cox regression model			
DCOG	***n***	**Hazard ratio**	**95% Confidence Interval**	***p***
TAL1	14	0.193	0.026-1.441	0.109
HOX11L2	17	2.048	0.814-5.149	0.128
HOX11	6	0.536	0.072-4.004	0.543
CALM-AF10	3	4.836	1.406-16.637	0.012
Rest	32	0.808	0.330-1.977	0.64
	B. Multi-variate analyses using Cox regression model			
DCOG	***n* (rel)**	**Hazard ratio**	**95% Confidence Interval**	***p***
TAL1$^+$	14 (1)	0.138	0.018-1.054	0.056
TAL1$^-$	58 (19)			
WBC		1.000	0.998-1.002	0.937
Age		1.086	0.978-1.206	0.123
Gender		0.349	0.099-1.230	0.102
HOX11L2$^+$	17 (7)	2.521	0.902-7.046	0.078
HOX11L2$^-$	55 (13)			
WBC		1.000	0.998-1.002	0.842
Age		1.091	0.966-1.231	0.16
Gender		0.446	0.127-1.571	0.209
CALM-AF10$^+$	3 (3)	3.831	1.062-13.816	0.04
CALM-AF10$^-$	69 (17)			
WBC		1.000	0.998-1.002	0.811
Age		1.039	0.931-1.160	0.491
Gender		0.519	0.146-1.840	0.31

(A) Univariate Cox proportional hazard analysis using disease-free survival for the various T-ALL subgroups. Hazard ratio for each specific molecular-cytogenetic subgroup is indicated relative to T-ALL cases lacking that molecular-cytogenetic aberration. (B) Multivariate Cox proportional hazard analysis using WBC, age, gender and disease-free survival as variables. Age at diagnosis is given in years; WBC at diagnosis is given as 10^9/L.

Immunophenotypic and molecular characterization of T-ALL subgroups

The various molecular-cytogenetically defined T-ALL subgroups were further characterized for immunophenotype and the expression of several transcription factors including *TAL1, LYL1, LMO1* and *LMO2,* which are frequently aberrantly expressed in T-ALL. The immunophenotypic characteristics of the various DCOG subgroups are summarized in Table 4. All *TAL1*-rearranged cases were CD2 and CD5 positive. The majority of patients (n=9) expressed surface membrane CD3 (SmCD3), TCR$\alpha\beta$ chains (n=9), and were CD4/CD8 double positive (n=7) or CD8 single positive (n=3). None of the patients was single positive for CD4. Nine out of ten cases tested expressed cytoplasmic-β (Cytβ). Five out of 14 samples from patients were TCR$\alpha\beta$ negative, and four of these samples tested for Cytβ expression did express Cytβ.

The *HOX11L2* subgroup was heterogeneous regarding the expression of immunophenotypic markers (Table 4). Twelve out of seventeen patients were SmCD3 negative, but five expressed SmCD3 of whom three also expressed TCRγδ but not TCR$\alpha\beta$. CD1 was expressed in seven samples, and CD10 also in seven samples with

three samples expressing both CD1 and CD10. CD4 or CD4/CD8 was expressed in ten and six patients, respectively. Four out of eleven samples tested expressed Cytβ. Five patients expressed CD34 (all CD1 negative), with three samples as well as another $CD1^-/CD34^-$ sample expressing the myeloid markers CD33/CD13, possibly reflecting immature cases.

In the *HOX11* subgroup, all six patients were positive for CD1, and three samples expressed CD10 consistent with an early cortical T-cell developmental stage. Four samples were CD4/CD8-double positive whereas two samples were CD4 single positive without evidence for TCRαβ, or TCRγδ expression. None of the four samples tested expressed Cytβ. All three *CALM-AF10*-positive patients expressed the myeloid expression markers CD33 and/or CD13. Two patients also expressed CD34. CD2 expression was found in one patient, whereas two patients expressed CD5. Only one sample expressed CD4. None of these patients expressed Cytβ, SmCD3 or TCR expression consistent with an early, prothymocytic developmental stage.

The expression levels of *TAL1, LYL1, LMO1* and *LMO2* were investigated in the cytogenetic subgroups using RQ-PCR. As expected, the *TAL1* expression level was highest in the *TAL1* subgroup and significantly higher than in the other T-ALL subgroups (Figure 2A). The range of *TAL1* expression of the *remaining*-group overlapped that of the *TAL1* subgroup. *TAL1* expression was also found in normal bone-marrow samples at levels equal to those in the *TAL1* subgroup. Expression of *LYL1* was not limited to immunophenotypically immature cases, and almost all samples expressed *LYL1* as previously reported.[11] The *TAL1* subgroup had the lowest *LYL1* expression, but significantly higher levels of expression were identified in the other cytogenetic and immunophenotypically immature subgroups (*CALM-AF10>HOX11L2>HOX11;* Figure 2B). *CALM-AF10*-positive samples and the *remaining* group expressed significantly higher levels of *LYL1* than did normal bone marrow control samples (MWU, p=0.001 for both subgroups). *TAL1* and *LYL1* were expressed in a reciprocal pattern, (Rs=-0.427, p<0.001). *LMO1* was equally expressed in about half of the samples of each subgroup, but all three *CALM-AF10*-positive samples were negative (Figure 2C). Levels of *LMO1* expression were higher in T-ALL samples than in normal bone marrow controls. In the *remaining*-group, the two samples with the highest levels of expression contained a translocation involving the *LMO1* gene. Both patients expressed *LMO1* at levels that were 3-1000 fold higher than those in other *LMO1* positive samples. *LMO2* expression was lowest in the *TAL1* subgroup, although the higher levels in the other subgroups were not statistically significantly different (Figure 2D). The *remaining*-group had the highest levels of *LMO2* expression, which were significantly higher than in the *TAL1* and *HOX11L2* subgroups and the normal bone marrow control group. *LMO2* expression levels were significantly correlated with *LYL1* expression levels (Rs=0.434; p<0.001), but not with *TAL1* expression levels (*not shown).*

Table 4. Expression of immunophenotypic markers for the various T-ALL subgroups

DCOG (n=72)	*CD34*	*CD33/ CD13*	*CD2*	*CD5*	*CD1*	*CD10*	*TdT*	*Cytβ*	*SmCD3*	*TCRαβ*	*TCRγδ*	*CD4*	*CD8*	*HLADR*
TAL1	2/14 (14)	0/14 (0)	13/13 (100)	14/14 (100)	5/14 (36)	2/14 (14)	14/14 (100)	9/10 (90)	9/14 (64)	9/14 (64)	0/14 (0)	7/14 (50)	10/14 (71)	0/14 (0)
HOX11L2	5/17 (29)	3/17 (18)	15/17 (88)	17/17 (100)	7/17 (41)	7/17 (41)	16/17 (94)	4/11 (36)	5/17 (29)	0/17 (0)	3/17 (18)	16/17 (94)	6/17 (35)	0/16 (0)
HOX11	0/6 (0)	0/6 (0)	6/6 (100)	6/6 (100)	6/6 (100)	3/6 (50)	6/6 (100)	0/4 (0)	1/6 (17)	0/6 (0)	0/6 (0)	6/6 (100)	4/6 (67)	0/6 (0)
CALM-AF10	2/3 (67)	3/3 (100)	1/3 (33)	2/3 (67)	1/3 (33)	1/3 (33)	2/3 (67)	0/2 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (33)	0/3 (0)	0/3 (0)
Remaining	9/32 (28)	3/32 (9)	25/32 (78)	32/32 (100)	11/32 (34)	11/32 (34)	26/32 (81)	7/22 (32)	13/32 (41)	8/32 (25)	2/32 (6)	18/32 (56)	18/32 (56)	2/32 (6)

All T-ALL samples were CD7 and cytoplasmic CD3 positive. Immunophenotypic marker expression is shown as the number of positive cases for which more than 25% of cells stained positive for the corresponding marker compared to the total amount of cases analyzed (percentages are given in brackets); Cytβ: cytoplasmic β; TCR, T-cell receptor; HLA-DR, human leukocyte antigen-DR; TdT, terminal deoxynucleotidyltransferase; SmCD3, surface membrane CD3; ND, not done.

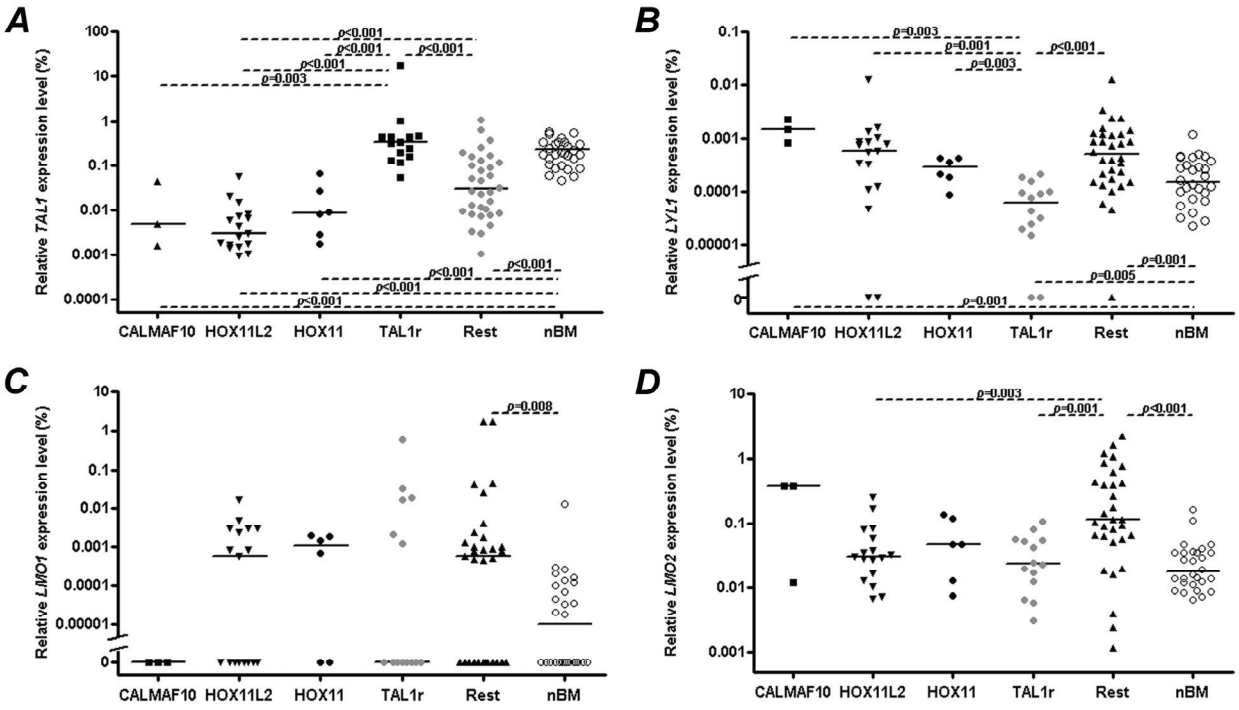

Figure 2. Expression levels of *TAL1, LYL1* and *LMO2* in T-ALL subgroups
Relative levels of expression of (A) TAL1, (B) LYL1, (C) LMO1 and (D) LMO2 as percentage of expression of the housekeeping gene GAPDH for the molecular-cytogenetically distinct T-ALL subgroups (CALM-AF10, HOX11L2, HOX11, TAL1, remaining group and bone marrow control samples). All p-values below p=0.0125 are considered statistically significant (Bonferonni significance level).

DISCUSSION

Using RQ-PCR and FISH we assigned seventy-two T-ALL samples to *TAL1, HOX11L2, HOX11,* or *CALM-AF10* subgroups or a *remaining*-group of patients lacking any of these abnormalities. The results of RQ-PCR closely matched those of FISH. One

patient expressed *HOX11L2* at levels equal to those in other patients with *HOX11L2*-rearrangements, patients, but did not have a FISH-detectable abnormality. Given the variability in breakpoints on chromosomes 5 and chromosome 14,[31-34] as well as the identification of the alternative *HOX11L2* translocation t(5;7)(q35;q21),[17,35] this patient may have a translocational variant not detected by our FISH approach. Based on the observed hybridization patterns within our cohort, we expect that further translocation variants may exist. Rearrangements involving the *TAL1* gene were found in about 20% of both cohorts of patients, which is consistent with the reported frequencies of 19-26% in other T-ALL series.[8,20,36,37] In line with some other studies,[12,20,21] *TAL1* rearrangements in the DCOG cohort tended to be associated with a better outcome, but did not predict for good outcome in the COALL cohort nor in a overall stratified analysis. It might be that therapy affected the prognostic relevance of *TAL1*-rearrangements in the two cohorts. Differences in follow-up duration and the limited number of patients in the two cohorts may further influence the results. Albeit not statistically significant, the *TAL1*-subgroup had the highest WBC in both cohorts, in line with previous observations.[12,20] The majority of patients in the *TAL1*-rearranged subgroup had an immunophenotypic signature corresponding to the TCR$\alpha\beta$ positive late cortical development stage[6,7,11] which, in turn, corresponds to the $\alpha\beta$-lineage T-ALL category of the TCR-based classification system by Macintyre and co-workers.[8,38] In our DCOG cohort, 5/14 *TAL1*-rearranged samples displayed a more immature immunophenotype consistent with an early cortical, pre-$\alpha\beta$ developmental stage.

Strikingly, only CD4/CD8-double positive or CD8-single positive samples but no CD4 single positive samples were present in the *TAL1* subgroup. Absence of CD4-single positive samples in the *TAL1* subgroup may reflect the oncogenic mechanism of *TAL1* in human T-ALL, as previously demonstrated in various mouse models.[39-41] The *TAL1* protein probably acts through an Id-like mechanism by binding and inhibiting E2A (E12/E47) and HEB helix-loop-helix transcription factors.[42] The E2A/HEB transcription factors normally bind to E-boxes located in promoter regions of various genes including *pre-Tα, TCRα, TCRβ*[40] and CD4.[41,43] Twenty-four percent of patients in the DOCG cohort had *HOX11L2*-rearrangements and/or high expression of *HOX11L2,* which is in accordance with the frequency found in other pediatric T-ALL patients.[8,12,33] In the DCOG cohort, there was a strong trend for an association between *HOX11L2* and poor outcome while for the COALL cohort, *HOX11L2* was a significant predictor of poor outcome. This is in line with some earlier studies,[11,17] but not other studies,[12,18,19] indicating that the prognostic relevance of *HOX11L2* could be dependent on the therapy given, although other suggestions have been raised. Most of the *HOX11L2*-positive cases in these latter studies were CD1-positive which is a prognostically favorable marker.[9,10,44] It was therefore argued that differences in the frequency of CD1 positive *HOX11L2*-subgroups in the various studies may underlie the differences in treatment outcome.[18,33] In our DCOG cohort, about 40% of the *HOX11L2*-rearranged patients expressed CD1. Three out of seven CD1-positive, *HOX11L2*-rearranged patients relapsed (43%) compared to four out of ten CD1-negative, *HOX11L2*-rearranged patients (40%), making it unlikely that conflicting results can be attributed to differences in CD1 expression. For the entire DCOG cohort, the outcome of CD1-positive patients did not differ significantly from that of

CD1-negative patients. Recently, it was demonstrated that a minority of *HOX11L2*-rearranged T-ALL patients also carry extra-chromosomal *NUP2I4-ABLI* amplifications.[45] One of the *HOX11L2*-rearranged patients in our DCOG cohort (patient #20) had a *NUP2I4-ABLI* positive subclone at diagnosis, comprising 5% of leukemic cells. Analysis of the relapse sample revealed that this subclone was responsible for the relapse (*unpublished data).* Differences in the prognostic relevance of *HOX11L2* abnormalities between several studies may therefore also depend on the presence of *ABLI* abnormalities. In a pilot screening, we also investigated whether the presence of activating mutations in *NOTCHI*, identified in about 50% of T-ALL patients,[46] could provide another explanation for the observed differences in outcome for *HOX11L2*-rearranged patients between various studies. Activated mutations in *NOTCH1*, located in the same domains as previously published, were identified in six out of eight *HOX11L2*-positive patients. Only two patients with *NOTCH1* mutations relapsed, whereas two additional *HOX11L2*-positive patients who relapsed had no evidence of *NOTCH1* mutations. This indicates that *NOTCH1* mutations cannot explain the observed differences in outcome between the various studies. In support of this, *NOTCH1* mutations were also identified in other cytogenetic subgroups including the *TAL1*, the *HOX11* and the *remaining* group. No association with outcome was observed between patients with or without *NOTCH1* mutations.

In correlation to immunophenotype, the differences in CD1 positivity between the *HOX11* and *HOX11L2* subgroups indicate that these subgroups are arrested at slightly different T-cell developmental stages and therefore may have different responses to therapy. The presence of other immunophenotypic markers supports the view that *HOX11*-rearranged T-ALL is arrested at a strictly early cortical[44,47] or immature-β (IMβ) developmental stage,[8,38] while *HOX11L2*-rearranged T-ALL comprises both immature and TCRγδ-positive mature cases.[8] Asnafi *et al.* concluded that the cells in *HOX11L2*-rearranged T-ALL resembled αβ-lineage cortical thymocytes that were differentiated towards unusual TCRγδ-expressing cells as intermediates between the αβ-lineage and the γδ-lineage.[8] The expression of TCRγδ in three of our patients supports this hypothesis.

In contrast to results of other studies,[12,14,15,17,19] we found that *HOX11* expression exactly matched with *HOX11* chromosomal rearrangements. 10q24 abnormalities had been observed in the karyotypic analysis for only three patients. In several studies, *HOX11* abnormalities have been associated with an excellent prognosis.[12-15] Due to the relatively low incidence of this abnormality in pediatric T-ALL patients in contrast to adult T-ALL patients,[8,15] it has not yet been validated whether children with these abnormalities have a favorable outcome.

Four percent of our T-ALL patients had *CALM-AF10* fusion transcripts detectable by PCR which is in agreement with the 2-5% positivity by karyotyping or FISH as described previously.[5,33] All three patients with *CALM-AF10* relapsed during therapy. The relapse sample of patient #38 confirmed the involvement of the *CALM-AF10* clone at relapse. Therefore, the presence of *CALM-AF10* may be associated with a poor outcome. Since we had only three positive cases in our current cohort, these data need further validation. The expression of the transcription factors *TAL1, LYL1, LMO1* and *LMO2,* frequently expressed in T-ALL without apparent chromosomal

abnormalities, was associated with T-cell differentiation status. In contrast to previous suggestions that *LYL1* is limited to rare immunophenotypic pro-thymocytic T-ALL cases, our results, in line with those of another study, indicate that *LYL1* is expressed in nearly all T-ALL cases,[11] but in striking inverse correlation with *TAL1* expression. *LYL1* expression is associated with early developmental stages and is especially expressed in *CALM-AF10*-positive cases followed by *HOX11L2* and *HOX11*-positive cases, while *TAL1* is highly expressed in cases that are associated with more advanced stages of differentiation. High *TAL1* expression was not only limited to *TAL1*-rearranged cases. These homologous helix-loop-helix transcription factors, *LYL1* and *TAL1*, may both act through an Id-like mechanism,[42] and substantial expression of *LYL1, TAL1* or both may offer a therapeutic opportunity independent of cytogenetic subtype. *LMO2* expression correlates with the expression of *LYL1*, as observed previously.[8,15] In the *remaining* group, the two samples that contained *LMO1* translocations expressed *LMO1* at levels that were 3-1000 fold higher than that of the other *LMO1* positive samples. The significance of *LMO1* expression remains unclear for the non-*LMO1* rearranged samples. However, the highest levels of *LMO1* expression were observed in the *TAL1* subgroup, in line with a synergistic role of *LMO1* in *TAL1*-driven oncogenesis.[39,40]

In conclusion, in the present study we show that the outcome for molecular-cytogenetic subgroups of T-ALL characterized by *HOX11-, HOX11L2-, TAL1-* or *CALM-AF10* abnormalities differs, with poor outcome for patients with *HOX11L2*-rearranged T-ALL. Our data also suggest that *CALM-AF10*-positive pediatric patients have a poor outcome but due to the relative scarcity of *CALM-AF10* positivity in children, validation of this finding will be difficult.

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The cryptic chromosomal deletion del(11)(p12p13) as a new activation mechanism of *LMO2* in pediatric T-cell acute lymphoblastic leukemia

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ABSTRACT

To identify new cytogenetic abnormalities associated with leukemogenesis or disease outcome, T-cell acute lymphoblastic leukemia (T-ALL) patient samples were analyzed by means of the array–comparative genome hybridization technique (array-CGH). Here, we report the identification of a new recurrent and cryptic deletion on chromosome 11 (del(11)(p12p13)) in about 4% (6/138) of pediatric T-ALL patients. Detailed molecular-cytogenetic analysis revealed that this deletion activates the *LMO2* oncogene in 4 of 6 del(11)(p12p13)-positive T-ALL patients, in the same manner as in patients with an *LMO2* translocation (9/138). The *LMO2* activation mechanism of this deletion is loss of a negative regulatory region upstream of *LMO2*, causing activation of the proximal *LMO2* promoter. *LMO2* rearrangements, including this del(11)(p12p13) and t(11;14)(p13;q11) or t(7;11)(q35;p13), were found in the absence of other recurrent cytogenetic abnormalities involving *HOX11L2, HOX11, CALM-AF10, TAL1, MLL,* or *MYC. LMO2* abnormalities represent about 9% (13/138) of pediatric T-ALL cases and are more frequent in pediatric T-ALL than appreciated until now.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is a high-risk malignancy of thymocytes, and accounts for 10% to 15% of pediatric ALL cases. T-ALL often presents with a high tumor-mass that is accompanied by a rapid progression of disease. About 30% of T-ALL cases relapse within the first years during or following treatment and eventually die.[1]

Genetic analyses of T-ALL have elucidated an enormous heterogeneity in genetic abnormalities including chromosomal translocations, deletions, amplifications, and mutations.[2] These abnormalities result in the aberrant expression of transcription factors such as the basic helix-loop-helix (bHLH) genes *MYC, TAL1 (SCL), TAL2, LYL1,* or *bHLHB1;* genes involved in transcriptional regulation such as the cysteine-rich LIM-domain–only genes *LMO1* or *LMO2;* or the Krüppel-like zinc-finger gene *BCL11B.* Abnormalities can also affect genes that are involved in embryonic development such as the homeodomain genes *HOX11/TLX1* and *HOX11L2/TLX3;* members of the *HOXA* cluster; as well as signaling molecules such as the tyrosine kinase *ABL1*[3-6] (reviewed in Raimondi[7] and Rubnitz and Look[8]). Other translocations lead to the formation of specific fusion products and include *CALM-AF10*[9] or *MLL* rearrangements. Mutational mechanisms may also enhance gene activity as, for example, activating mutations in the *NOTCH1* gene were recently identified in about 50% of human T-ALLs.[10]

LMO2 encodes a protein that participates in the transcription factor complex, which includes E2A, TAL1, GATA1, and LDB1 in erythroid cells.[11,12] Within this transcription complex, LMO2 mediates the protein-protein interactions by recruiting LDB1, whereas TAL1, GATA1, and E2A regulate the binding to specific DNA target sites.[13] This complex regulates the expression of several genes in various cellular backgrounds including *C-KIT,*[14] *EKLF,*[15] and *RALDH.*[16] In normal T-cell development, *LMO2* is expressed in immature CD4/CD8 double-negative thymocytes, and is down-regulated during T-cell maturation.[17,18] In various mouse models, ectopic expression of *LMO2* leads to clonal expansion of T cells, eventually leading to T-ALL development. *LMO2*-mediated leukemogenesis seems restricted to the T cell, as transgenic mice with constitutive expression of *LMO2* in all tissues develop malignancies involving the T-lineage only.[19-22] The long latency of leukemia in these mice suggests that T-ALL development requires secondary mutations in addition to the activation of *LMO2.*[22,23]

LMO2-driven oncogenesis in humans was suggested by its frequent involvement in the chromosomal translocations t(11;14)(p13;q11) and t(7;11)(q35;p13) in T-ALL, in which the *TCRA/D* or *TCRB* locus is fused to *LMO2.*[24,25] Direct proof came from the retroviral *IL2Rgc* gene therapy trial for X-linked severe combined immunodeficiency (X-SCID) patients, in which 2 patients developed T-ALL after retroviral insertions in the *LMO2* gene.[26,27]

In this study, we report the identification of a new recurrent and cryptic deletion (del(11)(p12p13)) in about 4% (6/138) of pediatric T-ALL patients. Detailed molecular-cytogenetic analysis revealed that this deletion activates the *LMO2* oncogene in 4 of 6 of these del(11)(p12p13)-positive T-ALL patients, mainly through deletion of negative regulatory sequences upstream of *LMO2.* The relation to other recurrent cytogenetic abnormalities, the immunophenotypic characteristics, and the clinical outcome of this new cryptic abnormality in pediatric T-ALL are discussed.

MATERIALS AND METHODS

Patient samples

Viably frozen diagnostic bone marrow or peripheral blood samples from 64 pediatric T-ALL patients and clinical and immunophenotypic data were provided by the Dutch Childhood Oncology Group (DCOG). Patients were considered positive if more than 25% of total cells were positive for a specific immunophenotypic marker. A second pediatric T-ALL cohort (n=74) was obtained from the German Co-operative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL). The patients' parents or their legal guardians provided informed consent to use leftover material for research purposes in accordance with the rules of the review board of Erasmus MC and the Declaration of Helsinki. Leukemic cells were isolated and enriched from these samples as previously described.[28] All resulting samples contained 90% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)-stained cytospins. Viably frozen T-ALL cells were used for DNA and RNA extraction, and a minimum of $5x10^6$ leukemic cells was lysed in Trizol reagent (Gibco BRL, Life Technologies, Breda, The Netherlands) and stored at -80°C. A total of $25x10^3$ leukemic cells was used for cytospin slides for fluorescence in situ hybridization (FISH) and stored at -20°C. For the preparation of metaphase slides, a minimum of $5x10^6$ leukemic cells was cultured for 72 hours in serum-free medium (JRH Biosciences, Lenexa, KS) in the presence of IL7 (10 ng/mL) and IL2 (10 ng/mL), and harvested according to standard cytogenetic techniques.

Genomic DNA isolation, RNA extraction, and cDNA synthesis

Genomic DNA and total cellular RNA were isolated according to the manufacturer's protocol, with minor modifications. An additional phenol-chloroform extraction was performed and the DNA was precipitated with isopropanol along with 1 µL (20 µg/mL) glycogen (Roche, Almere, The Netherlands). After precipitation, RNA pellets were dissolved in 20 µL RNAse-free TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0). The RNA concentration was quantified spectrophotometrically. Following a denaturation step of 5 minutes at 70°C, 1 µg RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 pM) and oligodT primers (20 nM). The RT reaction was performed in a total volume of 25 µL containing 0.2 mM of each dNTP (Amersham Pharmacia BioTech, Piscataway, NJ), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI), and 25 U RNAsin (Promega). Conditions for the RT reaction were 37°C for 30 minutes, 42°C for 15 minutes, and 94°C for 5 minutes. The cDNA was diluted to a final concentration of 8 ng/µL and stored at -80°C.

BAC array–comparative genomic hybridization (BAC array–CGH)

Bacterial artificial chromosome (BAC) array–CGH analysis was performed using a dye-swap experimental design to minimize false-positive results. Patient genomic DNA (2 µg) and male/female reference DNA (2 µg; Promega) were fragmented by sonification (VibraCell Model VC130; Sonics & Materials, Newtown, CT). DNA fragmentation was verified by agarose gel electrophoresis. Individual reference and experimental

samples were then purified using the QIAQuick polymerase chain reaction (PCR) clean-up kit (Qiagen, Valencia, CA). Labeling reactions with Cy5-dUTP and Cy3-dUTP (PerkinElmer, Wellesley, MA) were performed with 5 µg purified restricted DNA using the Bioprime labeling kit (Invitrogen, Paisley, United Kingdom) according to the manufacturer's instructions. The patient and reference DNA were combined, denatured, and applied to the 1-Mb GenomeChip V1.2 Human BAC arrays (2632 BAC clones spotted on a single array; Spectral Genomics, Houston, TX) according to the manufacturer's protocol. Hybridization and washing procedures were performed as recommended, and the slides were scanned on a GenePix 4000B Microarray Scanner (Axon Instruments, Union City, CA). Cy3 and Cy5 fluorescent intensities at each DNA spot were quantified using GenePix Pro 4.0 Microarray Image Analysis Software, and the data were subsequently imported into SpectralWare software (Spectral Genomics). Using this software, background intensities were subtracted and initial fluorescence ratios were log2 transformed. The ratios for each clone were subsequently plotted into chromosome-ideograms. At this stage, known large-scale copy number polymorphisms were not considered disease related.[29]

Oligo array-CGH

Oligo array-CGH analysis was performed, as previously described,[30] on the human genome CGH Microarray 44A (Agilent Technologies, Palo Alto, CA), which consists of approximately 40000 60-mer oligonucleotide probes that span both coding and noncoding sequences with an average spatial resolution of approximately 35Kb. Briefly, 10 µg genomic reference or patient DNA was digested with *Alu*I (20 U) and *Rsa*I (20 U) (Invitrogen) overnight at 37°C. Verification of DNA fragmentation, purification, and Cy-3 or Cy-5 labeling was as described under "BAC array–comparative genomic hybridization (BAC array-CGH)." Experimental and reference targets for each hybridization were pooled and mixed with 50 µg human Cot-1 DNA (Invitrogen), 100 µg yeast tRNA (Invitrogen), and 1x hybridization control targets (SP310; Operon Technologies, Alameda, CA) in a final volume of 500 µL in situ hybridization buffer (Agilent Technologies). The hybridization mixtures were denatured at 95°C for 3 minutes, preincubated at 37°C for 30 minutes, and hybridized to the array in a microarray hybridization chamber (Agilent Technologies) for 14 to 18 hours at 65°C in a rotating oven (Robbins Scientific, Mountain View, CA). The array slides were washed in 0.5 x SSC/0.005% Triton X-102 at room temperature for 5 minutes, followed by 5 minutes at 37°C in 0.1 x SSC/0.005% Triton X-102. Slides were dried and scanned using a 2565AA DNA microarray scanner (Agilent Technologies). Microarray images were analyzed using feature extraction software (version 8.1; Agilent Technologies), and the data were subsequently imported into array-CGH analytics software v3.1.28 (Agilent Technologies).

FISH procedure

BACs were obtained from BAC/PAC Resource Center (Children's Hospital, Oakland, CA). BAC DNA was isolated using DNA MiniPrep plasmid kit (Promega) and labeled with biotin-16-dUTP/digoxigenin-11-dUTP (Roche) by nick translation.[31] BAC clones RP11-646J21, RP11-98C11, and RP11-603J2 were used for the characterization of the

telomeric breakpoints of the del(11)(p12p13), whereas the centromeric breakpoints were localized using RP11-36H11, RP11-769M16, and RP11-465C16. *LMO2* translocations were identified using a split-FISH procedure with the *LMO2* flanking BAC clones RP11-646J21 and RP11-98C11. FISH analysis was performed on freshly prepared interphase and metaphase slides from methanol/acetic acid cell suspensions or cytospins stored at -20°C. Slides were pretreated by an RNase and pepsin treatment, fixed with acid-free formaldehyde, and denatured at 72°C. Probes were denatured (4 minutes at 72°C in 70% formamide/2x SSC) in the presence of a 100-fold excess of human Cot-1 DNA (Gibco BRL). Following preannealing at 37°C for 30 minutes, biotinylated probes were hybridized overnight at 37°C, and visualized by fluorescein avidin (Vector Laboratories, Burlingame, VT) and biotinylated anti–avidin D sandwich detection (affinity purified; Vector Laboratories).

The digoxigenin hybridization signal was detected using anti-digoxigenin-rhodamine (Boehringer Mannheim, Mannheim, Germany) and donkey anti-sheep Texas Red (Jackson ImmunoResearch Laboratories, Westgrove, PA). Cells were counterstained with DAPI/Vectashield mounting medium (Vector Laboratories). Fluorescence signals were visualized on a Zeiss Axioplan II fluorescence microscope (Zeiss, Sliedrecht, The Netherlands) equipped with double and triple bandpass filters for simultaneous visualization of rhodamine-TR/FITC/DAPI, as well as a Plan-Apochromat 100 X/1.40 NA oil iris objective lens. Images were captured by using PSI MacProbe 4.3 software (Applied Imaging, Newcastle-Upon-Tyne, United Kingdom). *TAL1, HOX11L2, HOX11,* and *MLL* chromosomal rearrangements or the *SIL-TAL1* deletion was determined using FISH kits (DakoCytomation, Glostrup, Denmark),[32] hybridized, and scored as described by the manufacturer. The *CALM-AF10* chromosomal rearrangement was detected using FISH as previously described.[32]

Genomic and cDNA PCR

The genomic breakpoint in T-ALL patient 1950 was determined by long-range PCR using forward primer 5'-GATGCCTTCCCTCATGTA-3' (intron 1 *RAG2)* and reverse primer 5'-CGCAGTGCCTAGAACAGT-3' (intron 1 *LMO2).* PCR reactions were performed using 200 ng genomic DNA (200 ng/µL), 10 pmol primers, 10 nmol dNTPs, 4mM $MgCl_2$, 1.25 U ampli*Taq* gold (Applied Biosystems, Foster City, CA) in 10 X PCR buffer II (Applied Biosystems) in a total volume of 50 µL. After the initial denaturation at 94°C for 10 minutes, PCR was performed for 15 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 68°C for 3 minutes followed by 15 cycles consisting of 95°C for 15 seconds, 60°C for 1 minute, and 68°C for 3 minutes (+10 s/cycle).

To identify the *RAG2-LMO2* fusion gene in T-ALL patient 1950, cDNA PCR (RT-PCR) was performed in the presence of forward primer 5'-GTGGGCAGTCAGTGAATC-3' (exon 1 *RAG2)* and reverse primer 5'-TGCAAGTTCAGGTTGAAA-3' (exon 2 *LMO2)* in a total volume of 50 µL. Following initial denaturation at 95°C for 10 minutes, reactions were amplified for 39 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Ligation-mediated PCR

Ligation-mediated PCR (LM-PCR) was performed as previously described.[33] Briefly, 1 µg patient (2846) and control (2720) DNA was digested with blunt-end restriction

enzymes (*Eco*RV, *Dra*I, *Pvu*II, and *Stu*I), and 50 pM of an adaptor was ligated to both ends of the restriction fragments. The ligation products were subjected to 2 rounds of PCR with nested adaptor-specific primers AP1 (5'-GCT AGATAC GAC TCA GTATAG-3') and AP2 (5'-TAT AGG CGC ACG AAC G-3') and nested *LMO2* intron 1–specific primers *LMO2*F (5'-CAG CCA CAT GGG TAG AAC-3') and *LMO2*F nested (5'-TGG CAT TAG GGT ATG GAA-3'). The band that differed in size from the expected band in the control patient, lacking the del(11)(p12p13), was excised from the gel and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and subjected to direct nucleotide sequencing.

Quantitative real-time RT-PCR (RQ-PCR)

Expression levels of *LMO2, TAL1, HOX11,* and *HOX11L2* transcripts and the *CALM-AF10* fusion product were quantified relative to the expression level of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* using real-time RT-PCR in an ABI 7700 sequence detection system (Applied Biosystems) as described previously.[28,34] The expression levels relative to the *GAPDH* housekeeping gene were calculated following the equation: relative expression level as percentage of GAPDH expression = $2^{-\Delta Ct}$ X 100%, whereby $\Delta Ct = Ct_{target} - Ct_{GAPDH}$.

Primer and probe combinations were designed using Oligo 6.22 software (Molecular Biology Insight, Cascade, CO) and were purchased from Eurogentec (Seraing, Belgium). Primers and probe had melting temperatures of 65°C to 66.5°C and 73°C to 75°C, respectively, and performed with 95% efficiency or higher as determined from slopes of standard curves. This allows direct normalization of the target reaction to *GAPDH* expression levels at the Ct level.[34] Primers and probe for the detection of the housekeeping gene *GAPDH* have been described previously.[28] For the detection of total *LMO2* transcripts derived from the *LMO2* distal promoter (upstream of exon 1), *RAG2-LMO2* fusion transcripts and transcripts derived from the *LMO2* proximal promoter (exon 3), the forward primer 5'-TTG GGGACC GCTACT T-3', and reverse primer 5'-ATG TCC TGT TCG CAC ACT-3' were used in combination with the probe 5'-(FAM)-AAG CTC TGC CGG AGA GAC TAT CT-3'. For the detection of distal *LMO2* transcripts and/or *RAG2-LMO2* fusion transcripts, forward primer 5'-TCA ACC TGA ACT TGC AGT AG-3' and reverse primer 5'-TCT CTC GGG AAG GTC TAT TT-3' were used in combination with the probe 5'-(FAM)-AAC CAG AGA CAG AGG GAA GCT G-3'. For *CALM-AF10,* 5' and 3' *CALM-AF10* fusion transcripts were detected in separate reactions using the *CALM-AF10* forward primer 5'-TTA ACT GGG GGA TCT AAC TG-3' in combination with the 5' fusion transcript reverse primer 5'-GCT GCT TTG CTT TCT CTT C-3' or the 3' fusion transcript reverse primer 5'-CCC TCT GAC CCT CTA GCT TC-3', both in combination with the common *CALM-AF10* probe 5'-(FAM)-CTT GGA ATG CGG CAA CAA TG-(TAMRA)-3'. For detection of *HOX11* expression levels, the forward primer 5'-CTC ACT GGC CTC ACC TT-3' and reverse primer 5'-CTG TGC CAG GCT CTT CT-3' were used in combination with the probe 5'-(FAM)-CCT TCA CAC GCC TGC AGATC-(TAMRA)-3'. For detection of *HOX11L2* expression levels, forward primer 5'-TCT GCG AGC TGG AAA A-3' and reverse primer 5'-GAT GGA GTC GTT GAG GC-3' were used in combination with probe 5'-(FAM)-CCA AAA CCG GAG GAC CAA GT-(TAMRA)-3'. For the detection of *TAL1* transcripts, the forward primer 5'-TGC CTT CCC

TAT GTT CAC-3' and reverse primer 5'-AAGATACGC CGCACAAC-3' were used in combination with probe 5'-(FAM)-CCT TCC CCC TAT GAG ATG GAG A-(TAMRA)-3'. The *SIL-TAL1* primers (ENF601, ENR664) and probe (ENP641) for the detection of a *SIL-TAL1* deletion were used as recommended by the Europe Against Cancer Program.[32]

Statistical analysis

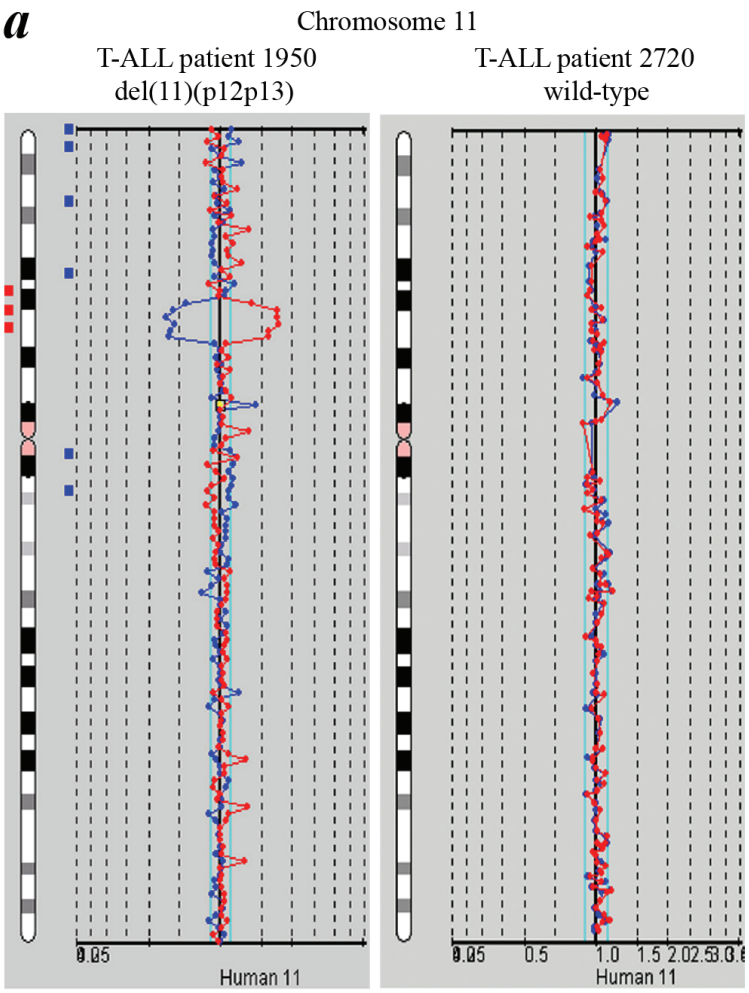
Kaplan-Meier curves were constructed in SPSS 11.0 software (SPSS, Chicago, IL) in a stratified analysis pairwise over strata, and *p* values were determined using the log-rank test. An event was defined as a relapse or nonresponse after induction therapy. The Mann-Whitney *U*-test was used to analyze differences in *LMO2* expression levels between subgroups. Data were considered statistically significant for *p* values less than 0.05.

RESULTS

New recurrent deletion del(11)(p12p13) in pediatric T-ALL

To identify new chromosomal abnormalities in pediatric T-ALL related to outcome and/or leukemogenesis, BAC array–CGH analysis was performed on a selected cohort of 30 of 64 clinically and karyotypically well-defined diagnostic T-ALL patient samples treated according to DCOG protocols. A recurrent loss of genomic material at chromosomal band 11p12-11p13 was found in 2 of 30 pediatric T-ALL cases (Figure 1A). Analysis of this pediatric T-ALL cohort and a second independent cohort (n=74) treated according to the COALL97 protocol using FISH confirmed the presence of the del(11)(p12p13) in both positive patients, but also revealed 4 additional patients with this same deletion (data not shown). BAC array-CGH analysis of these additional positive cases confirmed the presence of this del(11)(p12p13) (Figure 1B). This deletion is therefore present in about 4% (6/138) of pediatric T-ALL patients. In 4 patients (1950, 2846, 2104, and 10 110), the deleted region was flanked by the BAC clones RP1-187A11 (11p13) and RP11-72A10 (11p12), and comprised the clones RP1-22J9, RP11-90F13, RP11-91G22, AC090692.9, RP11-219O3, and RP11-36H11. In the 2 remaining cases (2774 and 704), the deleted area was smaller and flanked by the clones RP1-22J9 and RP11-72A10 (Figure 1B).

The resolution of the BAC array-CGH system as used for our analysis is approximately 1 Mb. To determine the exact telomeric and centromeric breakpoints for this del(11)(p12p13) in pediatric T-ALL, we used the oligo array–CGH system of Agilent Technologies with a resolution of approximately 35 Kb. In agreement with the BAC array-CGH data, the oligo array-CGH analysis showed identical genomic losses at 11p12-11p13 for these 6 patients albeit at higher resolution (Figure 2A-G).



1950	Normal	Loss	Loss	Loss	Loss	Loss	Normal
2846	Normal	Loss	Loss	Loss	Loss	Loss	Normal
2104	Normal	Loss	Loss	Loss	Loss	Loss	Normal
10110	Normal	Loss	Loss	Loss	Loss	Loss	Normal
2774	Normal	Normal	Loss	Loss	Loss	Loss	Normal
704	Normal	Normal	Loss	Loss	Loss	Loss	Normal

Figure 1. New recurrent deletion, del(11)(p12p13), in pediatric T-ALL (for colour figure see page 171)

(A) Chromosome 11 ideogram and corresponding BAC array-CGH plot of test DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for T-ALL patients 1950 (left panel) and 2720 (right panel). (B) Overview of BAC array–CGH results for the 11p12-11p13 region for the 4 DCOG and the 2 COALLT-ALL patients with del(11)(p12p13). The BAC clones present on the DNA array and located on chromosome bands 11p12-11p13 are shown. Specific genes located in this region are indicated. Depicted genome positions are based on the UCSC Genome Browser at http://genome.ucsc.edu/.

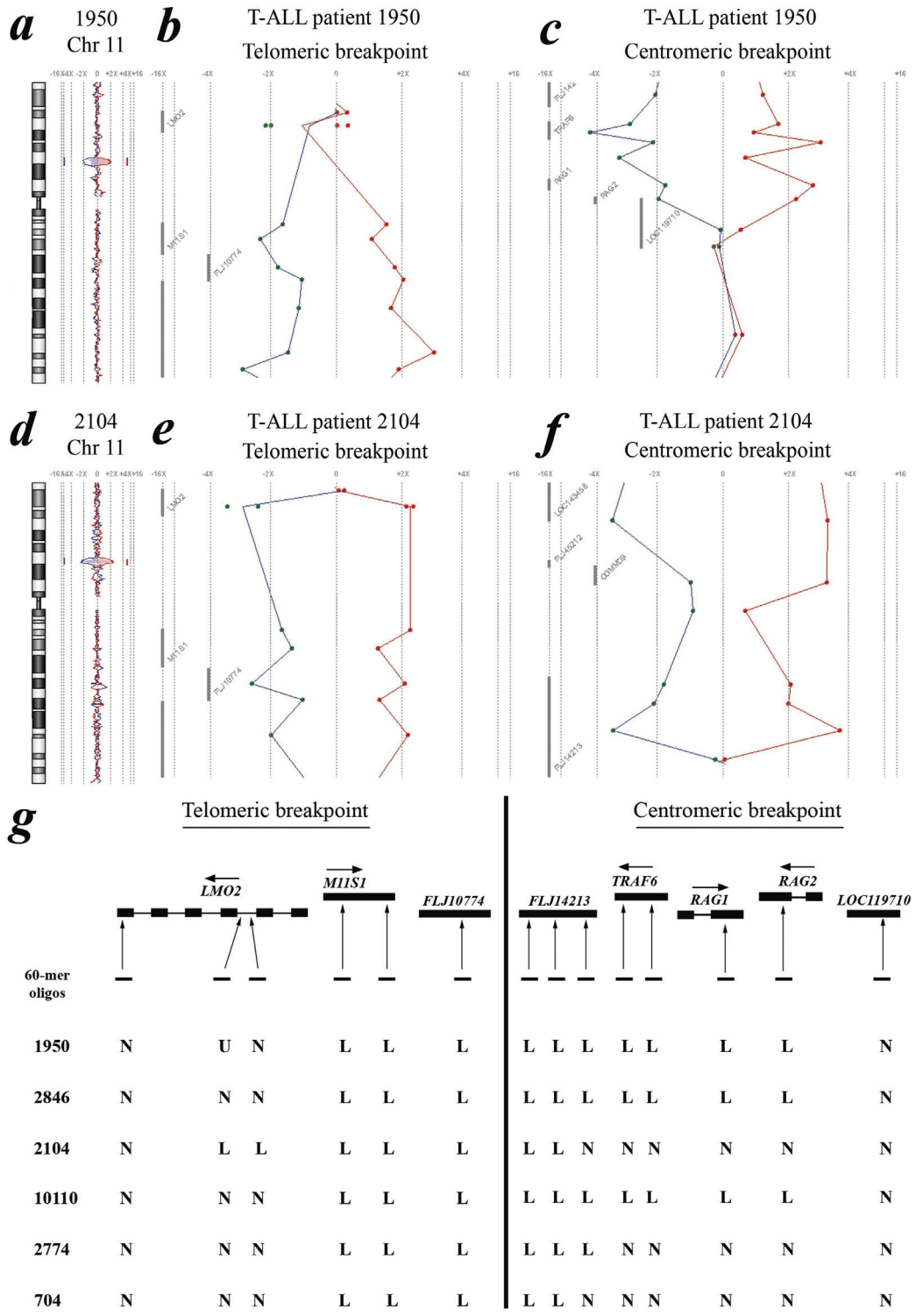


Figure 2. Molecular characterization of deletion, del(11)(p12p13), in 6 pediatric T-ALL patients
(for colour figures see page 172)

Chromosome 11 ideogram and corresponding oligo array–CGH plot of test DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for T-ALL patient 1950 (A) and patient 2104 (D). Hybridization signals in the absence of amplifications or deletions scatter around the "zero" line, indicating equal hybridization for patient and reference DNA. Hybridization signals around the -2X or +2X lines represent loss of the corresponding region in the patient DNA. Detailed analysis of the telomeric breakpoints in patients 1950(B) and 2104(E) and the centromeric breakpoints in patients 1950 (C) and 2104 (F) of the deletion, del(11)(p12p13). (G) Overview of oligo array-CGH results in the potential breakpoint regions for 4 DCOG and the 2 COALL T-ALL patients with del(11)(p12p13). The 60-mer oligos present on the DNA array and located in the telomeric and centromeric breakpoint regions, as well as the specific genes located in this region with their transcription direction, are shown. N indicates normal; L, loss; and U, noninformative.

Detailed analysis of the telomeric breakpoints indicated that both LMO2 probes located in intron 2 hybridized in a 1:1 ratio in patients 1950 (Figure 2B), 2846, 10 110, 2774, and 704 (Figure 2G), indicating that this part of LMO2 was retained. In all of these cases, both probes situated in M11S1 were deleted (Figure 2B,G). For patient 2104, the LMO2 intron 2 probes were lost, whereas the telomeric part of LMO2 (exon 6) was retained (Figure 2E), indicating that the genomic breakpoint is probably located downstream of LMO2 intron 2. At the centromeric breakpoint, the hybridization signals of both RAG1 and RAG2 probes were altered and indicated that one copy of both RAG1 and RAG2 genes was lost in patients 1950, 2846, and 10 110, whereas they had retained the LOC119710 locus (Figure 2C,G). For patients 2104 and 704, the centromeric breakpoint seemed to be situated in the FLJ14213 gene (Figure 2F-G). For patient 2774, both FLJ14213 probes were lost, whereas the TRAF6 probes hybridized normally.

FISH analysis confirms array-CGH data

To confirm the BAC and oligo array-CGH data and to further characterize the exact breakpoints of this del(11)(p12p13), metaphase and interphase cells of the positive T-ALL cases were analyzed by FISH. In Figure 3A, the genomic positions of the FISH probes are visualized. For patient 1950, FISH analysis with RP11-465C16, which covers both *RAG* genes, RP11-646J21, which covers the telomeric part of *LMO2,* and RP11-98C11, which is located directly centromeric of *LMO2,* confirmed heterozygous loss of a region directly upstream of *LMO2* (Figure 3B). The RP11-603J2 probe that includes part of the *LMO2* locus was partly retained in the mutant allele (Figure 3C), indicating that the telomeric breakpoint of the del(11)(p12p13) was situated in a 9-kb region surrounding exon 1 of *LMO2.* Similar analysis in this patient of the centromeric breakpoint indicated that both RP11-36H11 (Figure 3D) and RP11-769M16 (Figure 3E) were deleted, whereas at least part of RP11-465C16 was retained. This confirms that the telomeric breakpoint of the del(11)(p12p13) in this patient was located in or just flanking the *RAG* genes.

FISH analysis for the 5 other cases with del(11)(p12p13) (Table 1) confirmed that this deletion also targeted *LMO2* in patients 2846, 2104, and 10 110. However, for patients 2774 and 704 (Table 1) both RP11-603J2 and RP11-98C11 probes showed a normal hybridization pattern, suggesting that in these cases the break had occurred upstream of *LMO2* between the *LMO2* and *M11S1* genes.

A single patient from the DCOG T-ALL cohort had a classical t(11;14)(p13;q11) by conventional cytogenetics. To determine the exact frequency of classical t(11;14)(p13;q11) or the t(7;11)(q34;p13) translocations involving *LMO2,* both the DCOG and the COALL cohorts (n=138) were analyzed by FISH using *LMO2* flanking BAC clones (data not shown). In total, 9 cases were identified that contained a translocation involving *LMO2* (9/138, 6.5%), including the patient also positive by conventional cytogenetics. Including these *LMO2*-translocated patients, the frequency of *LMO2* rearrangements (t(11;14)(p13;q11), t(7;11)(q34;p13), or del(11)(p12p13)) was 9.4% (13/138) in total.

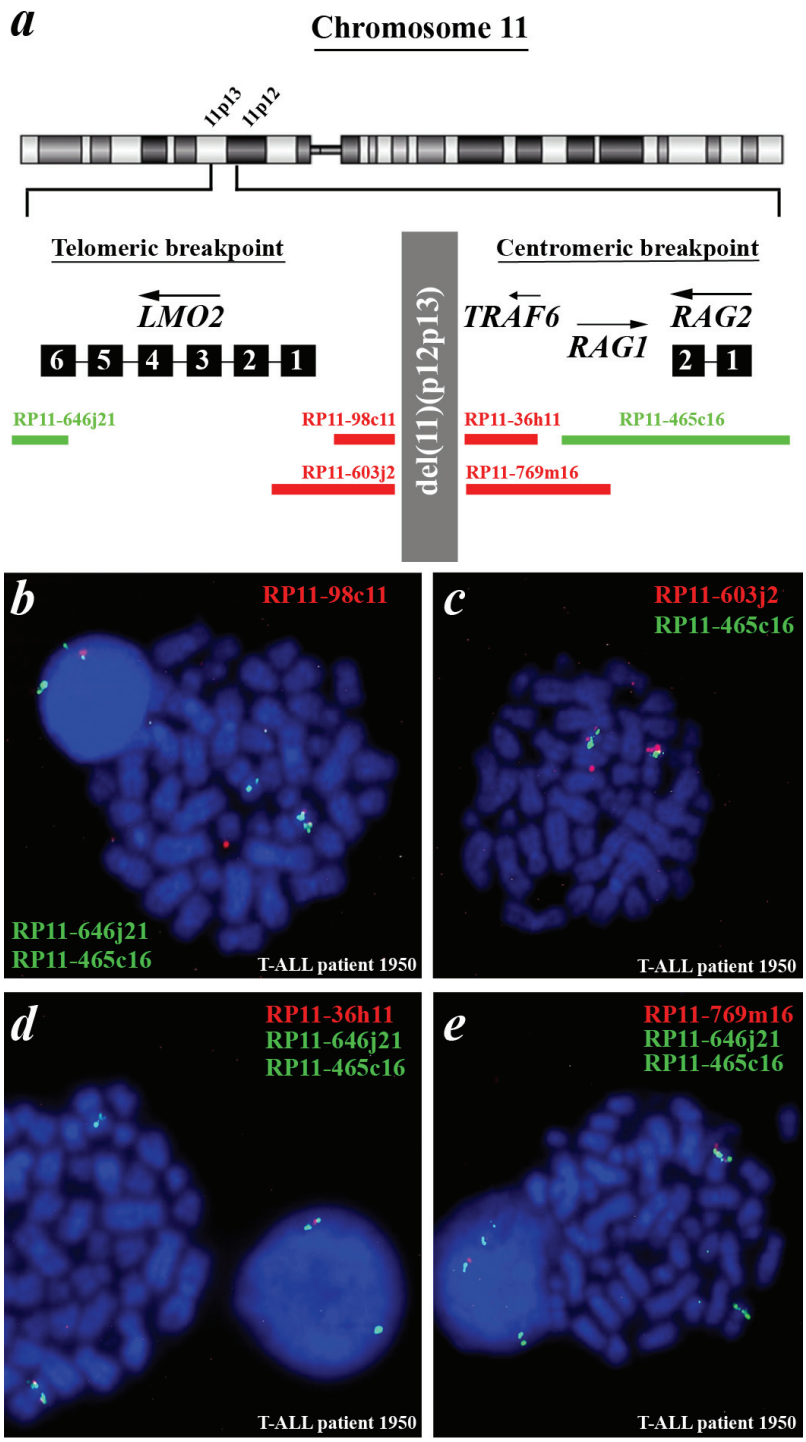


Figure 3. FISH analysis confirms the presence of del(11)(p12p13) in T-ALL patient 1950
(for colour figures see page 173)

(A) Chromosome ideogram and overview of the genomic position of the BAC clones used for FISH analysis, located in the telomeric and centromeric breakpoint regions. (B) Dual-color FISH analysis on metaphase spreads of patient 1950 using RP11-465C16 (green), RP11-646J21 (green), and RP11-98C11 (red). The wild-type allele of chromosome 11 shows 2 green and 1 red signal, whereas on the mutated allele the red signal is lost and both green signals fuse. The extrachromosomal red signal represents background. (C) Dual-color FISH analysis on metaphase spreads of the same patient using RP11-465C16 (green) and RP11-603J2 (red). The intensity of the red signal is lower compared with the wild-type allele of chromosome 11, suggesting that only part of RP11-603J2 is deleted. (D) Dual-color FISH analysis on metaphase spreads using RP11-465C16 (green), RP11-646J21 (green), and RP11-36H11 (red). The wild-type allele of chromosome 11 shows 2 green and 1 red signal, whereas on the mutated allele the red signal is lost and both green signals fuse. (E) Dual-color FISH analysis on metaphase spreads using RP11-465C16 (green), RP11-646J21 (green), and RP11-769M16 (red). The wild-type allele of chromosome 11 shows 2 green and 1 red signal, whereas on the mutated allele the red signal is lost and both green signals fuse.

Table 1. FISH analysis in 6 pediatric T-ALL patients with del(11)(p12p13)

	No. of hybridization signals					
	Telomeric breakpoint			Centromeric breakpoint		
Patient ID	**646j21**	**603j2**	**98c11**	**36h11**	**769m16**	**465c16**
1950	2	2*	1	1	1	2
2846	2	2*	1	1	1	2
2104	2	1	1	2*	2*	2
10110	2	2*	1	1	1	2
2774	2	2	2	2*	2*	2
704	2	2*	2	2*	2*	2

** intensity difference between the hybridization signal on the wild-type and the mutated allele.*

Molecular characterization of del(11)(p12p13)

We next characterized the genomic breakpoint of the del(11)(p12p13) in T-ALL patient 1950 in more detail. Long-range PCR analysis on genomic DNA using primers situated in intron 1 of *RAG2* and intron 1 of *LMO2* revealed a specific band of approximately 2000 bp (Figure 4A) for patient 1950 that was not present in a del(11)(p12p13)-negative control (2720). Sequence analysis showed the exact positions of the genomic breakpoints in both intron regions (Figure 4B). It was expected that this deletion gives rise to a fusion of exon 1 of *RAG2* to exon 2 of *LMO2*, which was confirmed at the mRNA level (Figure 4C-D). Subsequent RT-PCR failed to detect *RAG2-LMO2* fusion products in any of the remaining del(11)(p12p13)-positive T-ALL patients. Therefore, we performed ligation-mediated PCR (LM-PCR) in order to determine additional genomic breakpoints. In patient 2846, LM-PCR with an *LMO2* intron 1–specific primer revealed an aberrant PCR product in addition to the expected wild-type band (Figure 4E). Sequence analysis showed that in this case *LMO2* intron 1 sequences were fused to a region located 72-kb upstream of *RAG2* (Figure 4F).

Del(11)(p12p13) correlates with a mature immunophenotype and high *LMO2* expression in T-ALL

Immunophenotypic analysis of *LMO2*-rearranged cases revealed that patients with the del(11)(p12p13) did not express CD34, CD33, or CD1 but expressed mCD3 (Table 2). None of these cases expressed TCRγδ, whereas 2 patients expressed TCR$\alpha\beta$ (1950 and 2104) and 2 patients were CD4/CD8 double positive (1950 and 2846) in agreement with an immunophenotypic mature developmental stage. The *LMO2*-translocated patients were immunophenotypically more immature. Two of 3 cases expressed CD1, but none expressed mCD3 and/or the TCR. All 3 cases were CD4/CD8 double positive, consistent with an early cortical developmental stage.

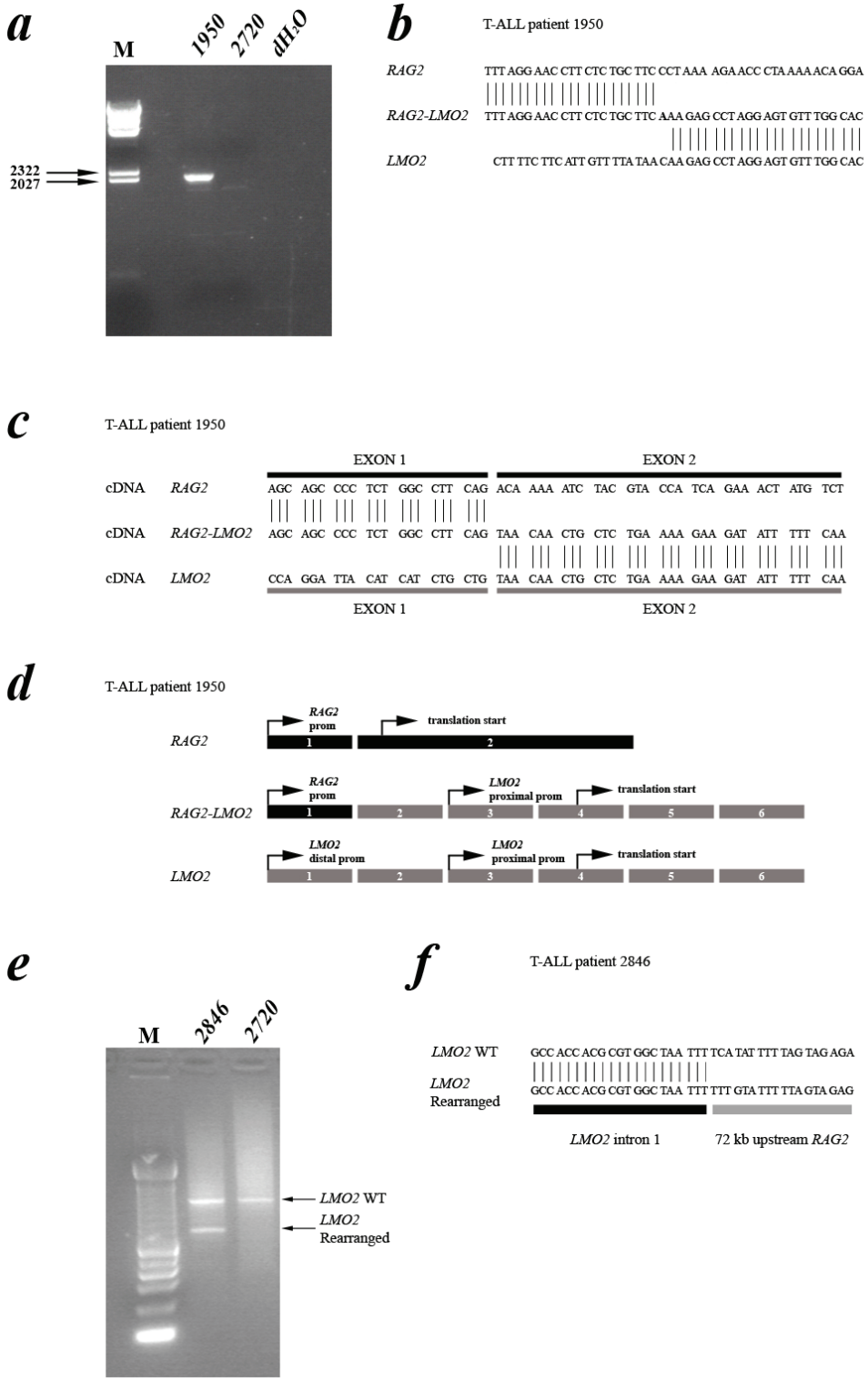


Figure 4. Molecular characterization of del(11)(p12p13) in T-ALL patients 1950 and 2846
(A) Long-range PCR analysis on genomic DNA of patient 1950 using primers situated in intron 1 of RAG2 and intron 1 of LMO2 revealed a specific band of approximately 2000 bp. Patient 2720 served as a negative control. (B) Sequence analysis confirmed the exact position of the genomic breakpoint. (C) PCR analysis on cDNA of this patient revealed a RAG2-LMO2 fusion gene, in which exon 1 of RAG2 was fused to exon 2 of LMO2. (D) Gene (exon) structure of both RAG2 and LMO2shows that the translation initiation sites are situated in exon 2 and exon 4, respectively. As a consequence, translation of the RAG2-LMO2 fusion gene will also be initiated in exon 4. (E) LM-PCR analysis on HincII-digested genomic DNA from patient 2846 using an LMO2 intron 1-specific primer revealed an aberrant PCR product of approximately 600 bp. The expected wild-type product is approximately 1000 bp and is visible in both patients 2846 and 2720, who served as a negative control. (F) Sequence analysis confirmed that in patient 2846 the LMO2 intron 1 sequences are fused to a genomic region upstream of RAG2. prom indicates promoter.

Table 2. Immunophenotypic characteristics of *LMO2-rearranged* pediatric T-ALL patients

		Positive cells (%)							
Patient ID	***LMO2* rearrangement**	**CD34**	**CD33**	**CD1**	**CD4**	**CD8**	**cytCD3**	**mCD3**	**TCRαβ**
1950	del(11)(p12p13)	0	0	0	85	91	90	80	Pos
2846	del(11)(p12p13)	0	10	0	48	57	75	26	Neg
2104	del(11)(p12p13)	0	0	0	6	1	98	45	Pos
2698	t(11;14)(p13;q11)	1	8	0	42	69	93	3	Neg
2789	t(11;14)(p13;q11)	1	12	25	59	7	85	12	Neg
2735	t(11;14)(p13;q11)	4	1	71	90	94	82	8	Neg

cytCD3 indicates cytoplasmic CD3; mCD3, membrane CD3; Pos, positive; and Neg, negative.

LMO2 mRNA expression levels of *LMO2*-rearranged versus non-rearranged cases were measured using RQ-PCR on 59 DCOG T-ALL patient samples for which immunophenotypic data were available. Since *LMO2* is highly expressed in T-ALL samples with an immature immunophenotype,[33] we divided T-ALL samples into 2 categories: the first category included the immature double-negative cases ($CD4^-$/$CD8^-$, $mCD3^-$, $Cyt\beta^-$), whereas the second category comprised more mature cases with evidence for TCRβ rearrangements ($Cyt\beta^+$) and/or TCR/CD3 expression.[35] For *LMO2* non-rearranged cases (WT), *LMO2* expression was significantly higher in the immature T-ALL cases compared with the immunophenotypically more advanced patients (Figure 5A, Mann-Whitney *U*, $p<0.001$). *LMO2*-rearranged cases had significantly higher *LMO2* levels compared with the *LMO2* non-rearranged T-ALL patients with a comparable immunophenotype ($p<0.001$). *LMO2* expression was low for patient 2774 (Figure 5A), which was in line with the observation that the deletion breakpoints did not affect the *LMO2* gene.

LMO2 rearrangements in relation to other oncogenic events in pediatric T-ALL

In order to determine the relation between *LMO2* rearrangements and other recurrent cytogenetic abnormalities in pediatric T-ALL, we screened all 13 *LMO2*-rearranged T-ALL patients for abnormalities at the *TAL1, HOX11L2, HOX11, CALM-AF10, MLL,* and *cMYC* loci using FISH analysis and RQ-PCR (data not shown). None of the *LMO2*-positive cases showed rearrangements of any of these loci. Nevertheless, in the del(11)(p12p13)-positive patient 2774 without involvement of *LMO2,* a *SIL-TAL1* interstitial deletion was identified. This indicates that del(11)(p12p 13)-positive T-ALL with elevated *LMO2* levels together with *LMO2*-translocated T-ALL samples reflect a separate cytogenetic subgroup without detectable *TAL1, HOX11L2, HOX11, CALM-AF10, MLL,* and *cMYC* abnormalities.

We further determined *TAL1* expression levels by RQ-PCR (Figure 5B). These analyses showed that for intermediate and mature T-ALL patients, *TAL1* is significantly more highly expressed in both *LMO2*-rearranged (Figure 5B, Mann-Whitney *U*, $p<0.001$) and *TAL1*-rearranged (Figure 5B, Mann-Whitney *U*, $p<0.001$) cases, compared with non-*LMO2/TAL1*-rearranged samples.

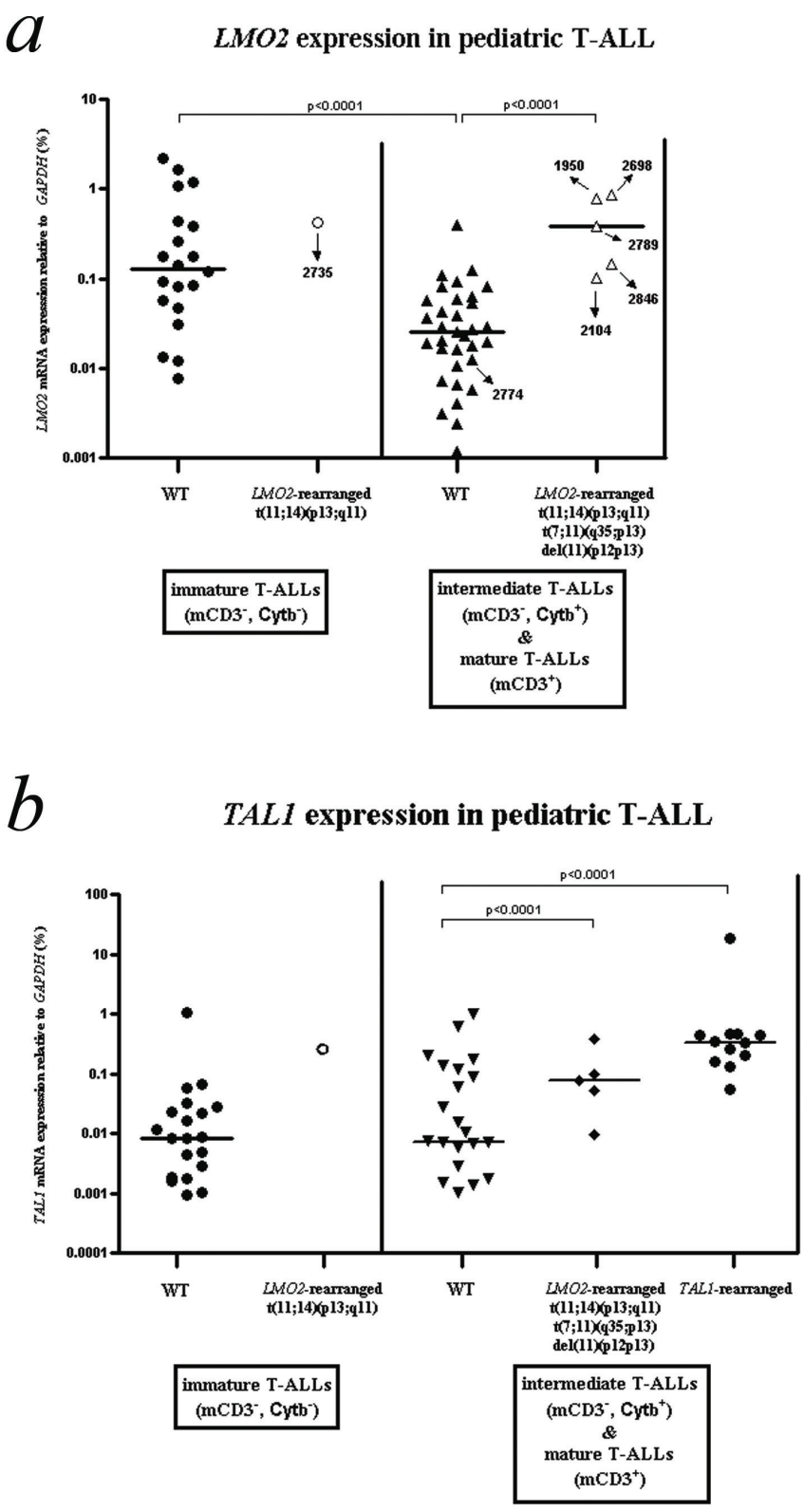


Figure 5. *LMO2* and *TAL1* expression in pediatric T-ALL
Relative expression levels of LMO2(A) and TAL1 (B) as percentage of GAPDH expression levels for 59 pediatric T-ALL patients (DCOG cohort). Patients were divided into 2 maturation stages according to their cytoplasmatic TCRβ (Cytβ) and membrane CD3 (mCD3) expression. Median expression levels are indicated by horizontal bars.

LMO2 activation induced by enhanced activity of the *LMO2* proximal promoter

In patient 1950, the del(11)(p12p13) resulted in a *RAG2-LMO2* gene fusion in which the distal *LMO2* promoter is replaced by the *RAG2* promoter (Figure 4C). However, a comparable fusion product was not detected in any of the remaining 3 del(11)(p12p13)-positive patients with elevated *LMO2* levels, suggesting that *RAG2-LMO2* fusion products were either expressed at very low levels or that other genomic regions were fused to *LMO2,* as found for patient 2846 (Figure 4F). We hypothesized that *LMO2* rearrangements due to the del(11)(p12p13) could result in the loss of a negative regulatory domain upstream of *LMO2,* with subsequent activation of the proximal promoter (exon 3), a situation comparable with *LMO2*-translocated patients.[36,37] To elucidate which kind of *LMO2* transcripts are predominantly expressed, we developed a double RQ-PCR: one RQ-PCR can quantify *LMO2* transcripts derived from the distal *LMO2* promoter as well as *RAG2-LMO2* fusion products. The second RQ-PCR quantifies total *LMO2* transcripts derived from both the distal and proximal *LMO2* promoters as well as *RAG2-LMO2* fusion products (Figure 6 and Table 3). These analyses revealed that *LMO2* transcripts derived from the distal promoter or *RAG2-LMO2* fusion products in del(11)(p12p13)-positive patients (1950 and 2846) represent only 5.5% to 9.3% of total *LMO2* transcripts (Figure 6 and Table 3). For both *LMO2*-translocated patients (2789 and 2735) also 8.5% to 10% of total *LMO2* transcripts originate from the distal promoter.

Clinical relevance of *LMO2* rearrangements in pediatric T-ALL

To study the prognostic relevance of *LMO2* rearrangements in pediatric T-ALL, Kaplan-Meier disease-free-survival (DFS) curves were created for *LMO2*-rearranged cases versus *LMO2* wild-type cases. In a stratified analysis of the combined DCOG and COALL cohorts (n=138), *LMO2* rearrangements had borderline significance for poor outcome (log-rank, p=0.03).

Table 3. Activation of the proximal promoter of *LMO2* in *LMO2*-rearranged T-ALL patients

	Total *LMO2 mRNA*		Long *LMO2* mRNA			
Patient ID, by mutation	**dC_t**	**Expression level**	**dC_t**	**Expression level**	**Long transcripts (%)**	**Proximal transcripts (%)**
Del(11)(p12p13)						
1950	6.14	0.58577	8.52	0.05421	9.3	90.7
2846	7.24	0.19499	10.15	0.01062	5.4	94.6
***LMO2* translocation**						
2789	6.81	0.29974	9.11	0.03005	10.0	90.0
2735	6.96	0.25799	9.43	0.02182	8.5	91.5

dCt indicates the expression of indicated promoter transcripts relative to the housekeeping gene GAPDH.

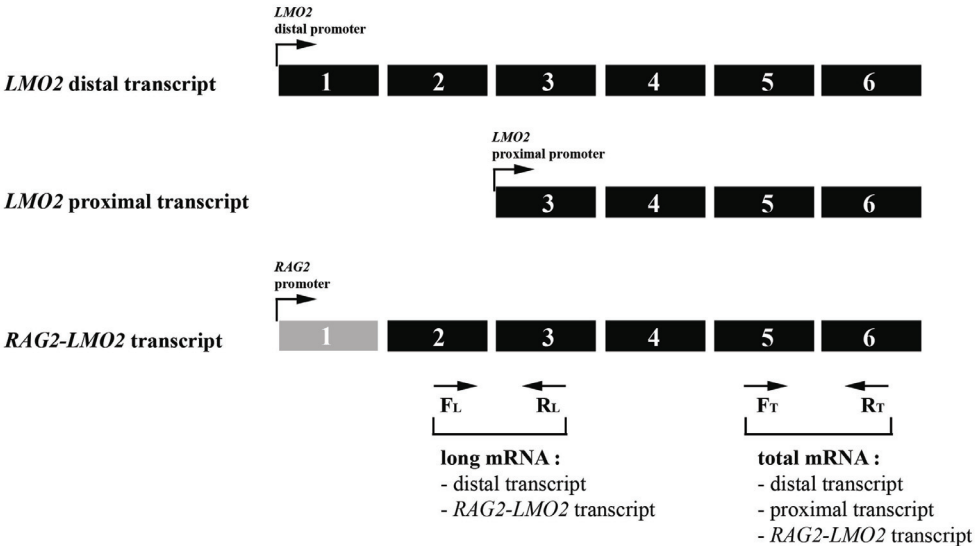


Figure 6. Elevated *LMO2* expression by activation of the *LMO2* proximal promoter
Relative expression of long and total mRNA transcript levels of LMO2 as measured by the RQ-PCR strategy. Long transcripts including the RAG2-LMO2 fusion transcript can be measured by the exon 2/3 primer combination, whereas the total amount of LMO2 transcript was measured using an exon 5/6 primer combination. Expression of proximal promoter transcripts is calculated by subtracting the long-transcript expression from the total expression. FL indicates forward primer long mRNA transcript; RL, reverse primer long mRNA transcript; FT, forward primer total mRNA transcript; and RT, reverse primer total mRNA transcript.

DISCUSSION

LMO2 has been identified as an oncogene in T-ALL due to its involvement in the translocation t(11;14)(p13;q11) or t(7;11)(q35;p13), in which the *TCR-LMO2* fusion results in a constitutive activation of the *LMO2* gene.[15,16] However, high *LMO2* expression levels have also been reported in the absence of translocations,[32,38] suggesting that alternative mechanisms may exist in T-ALL, resulting in the activation of *LMO2.*

Using the genome-wide array-CGH technique for the detection of genomic amplification and/or deletion areas, we identified a new recurrent deletion in pediatric T-ALL cases (i.e. the del(11)(p12p13)). Screening pediatric T-ALL samples showed that this deletion is present in about 4% of pediatric T-ALL patients (6/138 cases). The genomic breakpoints are located in intron 1 of *RAG2* and intron 1 of *LMO2* for patient 1950, placing *LMO2* under the control of the *RAG2* promoter. As expected, a *RAG2-LMO2* fusion product could be cloned. Since exon 1 of *RAG2* does not contain a translation initiation-site and the translation initiation-site of *LMO2* is located in exon 3, this fusion product will produce normal LMO2 protein. However, *RAG2-LMO2* fusions could not be identified in any of the remaining del(11)(p12p13)-positive T-ALL patients, suggesting that the localization of genomic breakpoints in these deletions is heterogeneous. This was demonstrated by the identification of the genomic breakpoint in patient 2846, in whom the deletion caused fusion of a genomic region approximately 72 Kb upstream of the *RAG2* gene with *LMO2* intron 1 sequences. Although the exact genomic breakpoints of both other del(11)(p12p13)-positive cases (2104 and 10110) remain to be identified, oligo array-CGH, FISH, and RQ-PCR

analyses predict involvement of *LMO2* in these cases in the same manner as in patients 1950 and 2846.

Recently, it has been proposed that deletion of negative regulatory sequences, located approximately 3000 bp upstream of exon 1 of *LMO2,* could contribute to ectopic *LMO2* expression in T-cell leukemia.[37] Of interest, this negative regulatory element was consistently removed in 4 del(11)(p12p13)-positive T-ALL cases that target *LMO2,* and may therefore provide a mechanism for the enhanced *LMO2* activation in pediatric T-ALL. However, based upon the *RAG2-LMO2* fusion product that was identified in patient 1950, promoter substitution could also contribute to elevated levels of *LMO2* expression. Our RQ-PCR data supported only marginal contribution of the distal *LMO2* promoter from the remaining wild-type allele or *RAG2-LMO2* fusion products to the total *LMO2* mRNA levels in del(11)(p12p13)-positive patients. Also, 2 *LMO2*-translocated patients as analyzed by RQ-PCR demonstrated low distal *LMO2* promoter activity, confirming enhanced proximal promoter activity due to the loss of this negative regulatory domain.[36,37]

Array-CGH and FISH data indicated that the deletion area for both del(11)(p12p13)-positive patients 2774 and 704 is smaller compared with the other 4 del(11)(p12p13)-positive patients. For both patients, the deletion seems to be located upstream of the negative regulatory region of *LMO2* as patient 2774 does not have elevated *LMO2* expression levels. These 2 cases may support the hypothesis that the minimally deleted region on chromosome 11 further contains a tumor-suppressor gene that could contribute to the pathogenesis of T-ALL.

LMO2-rearranged cases of the DCOG cohort including those with the del(11)(p12p13) as well as the 3 patients with a t(11;14)(p13;q11) expressed significantly higher levels of *LMO2* than *LMO2* non-rearranged T-ALL samples with a comparable immunophenotypic development stage (i.e. the cortical or mature T-cell developmental stage). The expression was comparable with immature T-ALL patients who highly express *LMO2* as a consequence of their immature developmental stage.[17,18] Nevertheless, a number of immunophenotypically more advanced T-ALL patients demonstrated high *LMO2* expression levels in the absence of currently known *LMO2* rearrangements, yet other alternative mechanisms leading to *LMO2* activation in pediatric T-ALL may exist.

LMO2-rearranged pediatric T-ALL samples with the del(11)(p12p13) may have a maturation stage that is more advanced compared with *LMO2*-translocated patients. Two of 3 cases with the del(11)(p12p13) involving the *LMO2* gene were TCR$\alpha\beta$ positive. In contrast, none of the *LMO2*-translocated patients expressed CD3 and/or TCR$\alpha\beta$, but 2 of these patients expressed CD1 conform an early cortical developmental stage. Whether this reflects true differences in maturation stage between patients with the del(11)(p12p13) and the *LMO2*-translocated cases needs to be validated in a larger panel of patients. Similar variations in cortical and mature T-cell developmental stages were also observed for *TAL1*-rearranged T-ALL patients in the same cohort.[32] Deregulation of *LMO2* or *TAL1* may lead to a similar T-cell developmental arrest. *TAL1* and *LMO2* act in the same pentameric transcription complex, and deregulation of either or both genes may lead to the (in)activation of identical target genes.

The frequency of *LMO2* rearrangements in both cohorts combined is about 9%, and includes 4 patients with *LMO2* rearrangements due to the del(11)(p12p13) and 9 cases with a t(11;14)(p13;q11) or the t(7;11)(q35;p13). These *LMO2* abnormalities were shown to be independent of other recurrent cytogenetic abnormalities including *TAL1, HOX11L2, HOX11, CALM-AF10, MLL,* or *cMYC.*[32] Patient 2774, who was del(11)(p12p13) positive but lacked *LMO2* activation, had an interstitial *SIL-TAL1* deletion.

Since *LMO2* and *TAL1* are frequently co-expressed in mature T-ALL cases and since no *TAL1* deletions and/or translocations were observed in the *LMO2*-rearranged cases, we determined *TAL1* mRNA expression in the 59 T-ALL samples for which *LMO2* expression data were available. These analyses confirmed that *TAL1* is significantly more highly expressed in both *LMO2*- and *TAL1*-rearranged T-ALL cases, compared with non-*LMO2/TAL1*-rearranged samples. These data further suggest that for del(11)(p12p13)-positive patients, alternative mechanisms of *TAL1* activation besides *TAL1* deletions and translocations may exist in T-ALL or that *TAL1* may be a direct target gene for *LMO2*-driven transcription.

The presence of *LMO2* rearrangements predicted for poor outcome in a stratified analysis of both the DCOG and COALL pediatric T-ALL cohorts. This prognostic significance has to be looked at cautiously due to the low number of patients, and a larger panel of T-ALL patients is needed to validate these findings. The presence of *LMO2* translocations did not predict for poor outcome in a previous study.[39]

In conclusion, we report the identification of a new cryptic cytogenetic abnormality (i.e. the del(11)(p12p13)) in 6 pediatric T-ALL patients targeting the *LMO2* gene in 4 cases. For del(11)(p12p13)-positive patients involving *LMO2,* the proximal *LMO2* promoter is highly activated due to the deletion of negative regulatory sequences upstream of *LMO2.* Abnormalities involving *LMO2,* including the del(11)(p12p13), the t(7;11)(q35;p13), and the t(11;14)(p13;q11), are more common in pediatric T-ALL (9%) as appreciated until now. *LMO2* abnormalities are independent from other recurrent cytogenetic abnormalities as frequently present in T-ALL.

ACKNOWLEDGMENTS

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3

The recurrent *SET-NUP214* fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia

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ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is mostly characterized by specific chromosomal abnormalities, some occurring in a mutually exclusive manner that possibly delineate specific T-ALL subgroups. One subgroup, including *MLL*-rearranged, *CALM-AF10* or inv(7)(p15q34) patients, is characterized by elevated expression of *HOXA* genes. Using a gene expression-based clustering analysis of 67 T-ALL cases with recurrent molecular genetic abnormalities and 25 samples lacking apparent aberrations, we identified 5 new patients with elevated *HOXA* levels. Using microarray-based comparative genomic hybridization (array-CGH), a cryptic and recurrent deletion, del(9)(q34.11q34.13), was exclusively identified in 3 of these 5 patients. This deletion results in a conserved SET-NUP214 fusion product, which was also identified in the T-ALL cell line LOUCY. SET-NUP214 binds in the promoter regions of specific *HOXA* genes, where it interacts with CRM1 and DOT1L, which may transcriptionally activate specific members of the *HOXA* cluster. Targeted inhibition of SET-NUP214 by siRNA abolished expression of *HOXA* genes, inhibited proliferation, and induced differentiation in LOUCY but not in other T-ALL lines. We conclude that SET-NUP214 may contribute to the pathogenesis of T-ALL by enforcing T-cell differentiation arrest.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is a thymocyte malignancy, and represents about 15% of pediatric patients with ALL. T-ALL often presents with a high tumor mass, accompanied by a rapid progression of disease. Still, about 30% of patients with T-ALL relapse during therapy or within the first 2 years following treatment and eventually die.[1]

Over the last few years, great progress has been made in unravelling the genetics of T-ALL, including recurrent chromosomal translocations (*TAL1, LYL1, LMO1, LMO2, HOX11*/*TLX1, HOX11L2*/*TLX3, MYB,* and *Cyclin D2),* deletions (*SIL-TAL1,* del(6q), del(9)(p21), and del(11)(p12p13)), amplifications (*NUP214-ABL1),* duplications (*MYB),* and mutations (*RAS* and *NOTCH1)*.[2-13] Some of these abnormalities are mutually exclusive and may delineate distinct T-ALL subgroups (i.e. *TAL1, LMO1, LMO2, HOX11, HOX11L2, CALM-AF10, MLL,* and Inv(7)). Others are shared by some of these subgroups and may lead to the deregulation of cell cycle (i.e. del(9)(p21) that includes the *CDKN2A/p15* and *CDKN2B/p16* loci).[3,4] Some may be acquired during leukemic growth, like the episomal *NUP214-ABL1* amplification.[6] *NOTCH1* activation mutations are present in more than half of all patients with T-ALL regardless of the presence of other rearrangements.[7] It has been hypothesized that activation of *NOTCH1* represents one of the most advanced abnormalities in T-ALL that may enable for uncontrolled proliferation and/or inhibition of apoptosis, possibly through up-regulation of the target genes *cMYC* and *DELTEX1*.[14-16]

In contrast to the wide variety of genetic abnormalities in T-ALL, initial microarray studies have revealed only 5 different expression clusters: immature/*LYL1*, *TAL1, HOX11, HOX11L2,* and *HOXA* clusters.[8,17] One of the explanations for this phenomenon is that patients with different molecular-cytogenetic defects may share a highly similar expression profile and are being recognized as one single expression cluster.[8,17] For example, patients with different abnormalities demonstrate high expression of genes of the *HOXA* cluster (*HOXA5, -A9, -A10,* and *-A11).* This cluster includes patients with *CALM-AF10*[8,18] or *MLL* rearrangements,[8,19] or patients with an inversion on chromosome 7 due to the rearrangement of the T-cell receptor-beta (*TCRβ)* locus into the *HOXA* cluster.[8,10] Elevated *HOXA* gene expression levels have also been reported in the absence of these genetic aberrations,[8,20] suggesting that alternative mechanisms of *HOXA* activation may exist in T-ALL.

Previously, we have studied the incidence and prognostic relevance of recurrent molecular-cytogenetic abnormalities for pediatric T-ALL.[21] Within our cohort, about half of the patients with T-ALL lack currently known molecular-cytogenetic abnormalities. To identify the underlying genetic defects in these patients, we used various high-resolution genomic screening strategies, including microarray-based comparative genomic hybridization (array-CGH). We recently described a new and recurrent deletion (i.e. the del(11)(p12p13)), in about 4% of patients with T-ALL.[12] This interstitial deletion leads to the loss of a negative regulatory domain upstream of *LMO2,* resulting in ectopic expression of this oncogene. Array-CGH also led to the identification of a recurrent duplication of *MYB* in about 10% of patients with T-ALL.[2,9]

In this study, we combined gene expression profiling and array-CGH analysis to

detect a new and recurrent molecular-cytogenetic abnormality in patients with T-ALL that co-clustered with 5 well-defined *HOXA*-activated T-ALL samples. We describe the cloning of a recurrent *SET-NUP214* fusion product in these samples, and identified a potential mechanism by which *SET-NUP214* may activate the *HOXA* gene cluster as potential leukemogenic event in T-ALL.

METHODS

Patient samples

Viably frozen diagnostic bone marrow or peripheral blood samples from 92 pediatric patients with T-ALL and clinical and immunophenotypic data were provided by the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL) and the Dutch Childhood Oncology Group (DCOG). The patients' parents or their legal guardians provided informed consent to use leftover material for research purposes in accordance with the Declaration of Helsinki. The Review Board of the Erasmus Medical Center approved the use of human participants in this study. Leukemic cells were isolated and enriched from these samples as previously described.[12] All resulting samples contained 90% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa-stained cytospins (Merck, Darmstadt, Germany). Viably frozen T-ALL cells were used for DNA and RNA extraction, and a minimum of $5x10^6$ leukemic cells were lysed in Trizol reagent (Invitrogen, Life Technologies, Breda, The Netherlands) and stored at -80°C. A total of $25x10^3$ leukemic cells was used to prepare cytospin slides for fluorescence in situ hybridization (FISH) and stored at -20°C.

Genomic DNA isolation, RNA extraction, and cDNA synthesis

Genomic DNA and total cellular RNA were isolated using Trizol (Invitrogen) according to the manufacturer's protocol, with minor modifications. An additional phenol-chloroform extraction was performed, and the RNA was precipitated with isopropanol along with 1 µL (20 µg/mL) glycogen (Roche, Almere, The Netherlands). After precipitation, RNA pellets were dissolved in 20 µL RNAse-free TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The RNA concentration was quantified spectrophotometrically. Following a denaturation step of 5 minutes at 70°C, 1 µg RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 µM) and oligodT primers (20 nM). The reverse transcriptase (RT) reaction was performed in a total volume of 25 µL containing 0.2 mM of each dNTP (Amersham Pharmacia Biotech, Piscataway, NJ), 200 U Moloney murine leukemia virus (M-MLV) RT (Promega, Madison, WI), and 25 U RNAsin (Promega). Conditions for the RT reaction were 37°C for 30 minutes, 42°C for 15 minutes, and 94°C for 5 minutes. The cDNA was diluted to a final concentration of 8 ng/µL and stored at -80°C.

Gene expression array analysis

Integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA). Copy DNA and ccRNA syntheses from total RNA, hybridization of

Humane Genome U133 plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA), and washing steps were performed according to the manufacturers' protocols. Probeset intensities were extracted from CEL files using GeneChip Operating Software (GCOS) version 1.4.0.036 (Affymetrix), and all arrays had a 3' end to 5' end GAPDH ratio lower than 3-fold. Probe intensities were normalized using the variance stabilization procedure (Bioconductor package VSN[22]; http://www.bioconductor.org/) in the statistical data analysis environment *R,* version 2.2.0 (http://www.r-project.org/). Differentially expressed genes between T-ALL subgroups were calculated using a Wilcoxon statistical test, and corrected for multiple testing error according to the false discovery rate procedure as developed by Hochberg and Benjaminin[23] using the Bioconductor package Multtest. The fold change was calculated using the formula: $e^{(\text{median value group A - median value group B})}$. Supervised clustering and principal component analyses were performed using GeneMath XT 1.6.1 software (Applied Maths, Austin, TX).

Array-CGH

Array-CGH analysis was performed, as previously described,[12,24] on the human genome CGH Microarray 44A (Agilent), which consists of approximately 40000 60-mer oligonucleotide probes that span both coding and noncoding sequences with an average spatial resolution of approximately 35 kb. Briefly, 10 µg genomic reference or patient DNA was digested overnight at 37°C with *Alu*I (20 U) and *Rsa*I (20 U; Invitrogen). Reference and patient DNA were purified and labeled with Cy5-dUTP and Cy3-dUTP (PerkinElmer, Wellesley, MA). Reference and patient DNA for each hybridization were pooled and mixed with 50 µg human Cot-1 DNA (Invitrogen), 100 µg yeast tRNA (Invitrogen), and 1x hybridization control targets (SP310; Operon Technologies, Alameda, CA) in a final volume of 500 µL in situ hybridization buffer (Agilent). The hybridization mixtures was denatured at 95°C for 3 minutes, preincubated at 37°C for 30 minutes, and hybridized to the array in a microarray hybridization chamber (Agilent) for 14 to 18 hours at 65°C in a rotating oven (Robbins Scientific, Mountain View, CA) at 20 rpm. The array slides were washed in 0.5x SSC/0.005% Triton X-102 at room temperature for 5 minutes, followed by 5 minutes at 37°C in 0.1x SSC/0.005% Triton X-102. Slides were dried and scanned using a 2565AA DNA microarray scanner (Agilent). Microarray images were analyzed using feature extraction software (version 8.1; Agilent), and the data were subsequently imported into array-CGH analytics software v3.1.28 (Agilent). Microarray data are available at http://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE 10609).

FISH

FISH analysis was performed on thawed cytospin slides using the LSI BCR-ABL ES translocation probe, according to the manufacturer's protocol (Vysis, Downers Grove, IL). Cells were counterstained with DAPI/ Vectashield mounting medium (Vysis). Fluorescence signals were visualized with a Zeiss Axioplan II fluorescence microscope (Zeiss, Sliedrecht, The Netherlands) using Mac Probe Software (version 4.3, Applied Imaging, Newcastle upon tyne, United Kingdom). The combined presence of a clonal

del(9)(q34.11q34.13) and an episomal *NUP214-ABL1* amplification in patient no. 120 was determined by FISH analysis as previously described[12] using bacterial artificial chromosomes (BACs) clones RP11-83J21 (covering *ABL1)* and RP11-618A20 (covering *ASS1,* located in the deleted area between *SET* and *ABL1).* BACs were obtained from BAC/PAC Resource Center (Children's Hospital, Oakland, CA).

RT-PCR and RQ-PCR

The *SET-NUP214* fusions were determined by RT–polymerase chain reaction (PCR) using forward primer 5'-TTCCCGATATGGATGATG-3' (exon 7 *SET)* and reverse primer 5'-CTTTGGGCAAGGATTTG-3' (exon 20 *NUP214).* PCR reactions were performed using 40 ng cDNA (8 ng/µL), 10 pmol primers, 10 nmol dNTPs, 4 mM $MgCl_2$, 1.25 U ampli*Taq* gold (Applied Biosystems, Foster City, CA) in 10x PCR buffer II (Applied Biosystems) in a total volume of 50 µL. After the initial denaturation at 94°C for 10 minutes, PCR was performed for 39 cycles of 95°C for 15 seconds, 60°C for 1 minute, and 68°C for 3 minutes. *NUP214-ABL1* fusions were determined as previously described.[6]

Expression levels of *HOXA, SET,* and *SET-NUP214* transcripts were quantified relative to the expression level of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* using quantitative RT-PCR (RQ-PCR) in an ABI 7700 sequence detection system (Applied Biosystems).

The *HOXA* primers were as described previously.[8] For *SET* expression, the forward primer 5'-TTCCCGATATGGATGATG-3' (exon 7 *SET)* and the reverse primer 5'-CCCCCCAAATAAATTGAG-3' (exon 8 *SET)* were used. For *SET-NUP214* expression, the primers used were as described in "RT-PCR and RQ-PCR."

Cell culture

T-ALL cell lines (DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Integro, Zaandam, The Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone (Invitrogen) and grown as suspension cultures at 37°C in humidified air containing 5% CO_2. LOUCY and SKW3 cells (10^7) were transfected with 50 nM *SET* siRNA by electroporation in 400 µL RPMI 1640 with L-alanyl-L-glutamine (Invitrogen) in 4 mm electroporation cuvettes (BioRad, Hercules, CA). The SET siRNAs were located in exon 5 (5'-GAAAUCAAAUGGAAAUCUGGAAA-3'), exon 3 (5'-CGAGUCAAACGCAGAAUAA-3'), and exon 8 (5'-AGGAGAAGAUGACUAAAUA-3'). To compensate for the amount of cell death induced as a consequence of the electroporation procedure, control cells were electroporated without siRNA. Electroporation was performed using an EPI 2500 gene pulser (Fischer, Heidelberg, Germany) applying a rectangle pulse of 350 V for 10 ms. After incubating for 15 minutes at room temperature, the cells were diluted 10-fold with RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Integro), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.125 µg/mL fungizone (Invitrogen) and incubated at 37°C and 5% CO_2. Cell viability was assessed by annexinV/propidium iodide (PI) staining and determined by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA). Electroporation of a fluorescein isothiocyanate (FITC)-labeled siRNA (Eurogentec,

Seraing, Belgium) and subsequent fluorescence-activated cell sorter (FACS) analysis indicated that transfection efficiencies were greater than 90%. Electroporation of this FITC-labeled siRNA also served as negative siRNA control.

Immunophenotyping and cell-cycle analysis was performed by multicolor flow cytometry using an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ). A total of 2 different 6-color labeling combinations were performed: labeling 1, anti-TCR$\alpha\beta$ (FITC), anti-TCR$\gamma\delta$ (peridine chlorophyll protein [PE]), CD3 (phycoerythrin [PerCP]), CD4 (PE-Cy7), CD7 (allophycocyanin [APC]), and CD8 (APC-Cy7); labeling 2, CD3 (FITC), CD10 (PE), CD45 (PerCP), CyCD3 (PE-Cy7), CD5 (APC), and CD2 (APC-Cy7). Data analysis was performed using FACSDiva software version 4.1.2 (BD Biosciences).

Protein extraction and Western blot analysis

Cell pellets stored at -80°C were briefly thawed and resuspended in 50 µL lysis buffer composed of 50 mM Tris buffer, 150 mM NaCl, 100 mM EDTA, 1% Triton X-100, 2 mM PMSF, 3% aprotinine (Sigma, Zwijndrecht, The Netherlands), 4 g/mL pepstatin (Sigma), and 4 µg/mL leupeptin (Sigma). Accordingly, cells were lysed for 15 minutes on ice. The supernatant of the lysed cells was cleared by centrifugation for 15 minutes at 13000*g* and 4°C. The protein content of the cleared lysates was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL) with different concentrations of bovine serum albumin as standards. Cell lysates containing 25 µg protein were separated on 10% polyacrylamide gels topped with 4% stacking gels, and transferred onto nitrocellulose membranes (Schleichler & Schuell, Dassel, Germany). Western blots were probed with mouse anti-SET (provided by K.N.) or with mouse antiactin (Sigma) antiserum. Anti-SET was used in different concentrations for proper detection of both SET (1:1000) and SET-NUP214 (1:250). Accordingly, the blots were labeled with peroxidase-conjugated anti-mouse IgG antibodies (DAKO, Glostrup, Denmark) and visualized using SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology).

IP and ChIP

For chromatin immunoprecipitation (ChIP) analysis, $20x10^6$ cells were crosslinked using formaldehyde to a final concentration of 1% for 15 minutes at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M followed by 5 minutes of incubation at room temperature. Fixed cells were washed twice using ice-cold 1x PBS and harvested in SDS buffer (100 mM NaCl, 50 mM Tris-HCl [pH 8.1], 5 mM EDTA [pH 8.0], 0.2% NaN_3, and protease inhibitors). After centrifugation, the pellet was resuspended in immunoprecipitation (IP) buffer (100 mM Tris [pH 8.6], 0.3% SDS, 1.7% Triton X-100, and 5 mM EDTA), and the cells were sonicated yielding genomic DNA fragments with a size of 500 to 1000 bp. After preclearing the lysates with protein A beads (50% slurry protein A-Sepharose; Upstate, Charlottesville, VA), the samples were immunoprecipitated overnight at 4°C with affinity-purified anti-NUP214 antibodies (provided by M.F.[25]), antidimethyl H3K79 (Upstate), antiacetylated H3 (Upstate) or anti-FLAG (Sigma). The immune complexes were recovered by adding 50 µL protein A beads and were incubated for 2 hours at

4°C. Subsequently, beads were washed with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], and 500 mM NaCL), LiCl buffer (250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 10 mM Tris-HCL [pH 8.0], and 0.2% NaN_3), and 1x TE buffer (10 mM Tris-HCL [pH 8.0], and 1 mM EDTA). The immune complexes were eluted from the beads by adding elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 15 minutes at room temperature. Cross-links were reversed by overnight incubation at 65°C. The eluted material was phenol/chloroform-extracted and ethanol-precipitated. The immunoprecipitated DNA was quantified by RQ-PCR using *HOXA*-specific promoter primers as previously described.[26]

For IP analysis, cells were washed twice using ice-cold 1x PBS and lysed in a single-detergent lysis buffer (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES [pH 7.0], 1 mM EDTA, 1% NP-40, and protease inhibitors). After preclearing the lysates with protein A beads (Upstate), samples were immunoprecipitated overnight at 4°C with rabbit anti-NUP214, rabbit anti-PP32 (gift from Dr. J. Brody, Johns Hopkins University, Baltimore, MD), mouse anti-SET, rabbit anti-CRM1 (gift from Dr. M. Yoshida, University of Tokyo, Japan), and rabbit anti-hDOT1L (gift from Dr. Yi Zhang, Lineberger Cancer Center, Chapel Hill, NC). The immune complexes were recovered by adding 50 µL protein A beads and were incubated for 2 hours at 4°C. Subsequently, beads were washed twice by single-detergent lysis buffer and twice by single-detergent lysis buffer without NP-40. The pellets were resuspended in loading buffer and boiled for 5 minutes, and Western blot analysis was performed as described in "Protein extraction and Western blot analysis."

RESULTS

Gene expression profiling of pediatric T-ALL subgroups

We have used gene expression profiling data to cluster 92 patients with T-ALL: 67 patients with known cytogenetic abnormalities and 25 patients without recurrent aberrations (from this point denoted as unknown patients). For the 67 patients with T-ALL who have one of the major molecular-cytogenetic abnormalities (i.e. *TAL1* [n=24], *LMO2* [n=9], *HOXA* [n=5], *HOX11/TLX1* [n=7], and *HOX11L2/TLX3* [n=22]), differentially expressed probesets were calculated from Affymetrix U133 plus 2.0 data based upon a Wilcoxon analysis and corrected for multiple testing for each probeset. Significant and differentially expressed probesets were obtained for the *TAL1, HOX11,* and *HOX11L2* subgroups (Figure 1A). No significant probesets were obtained for the *HOXA* subgroup or the *LMO2* subgroup (Figure 1A). As *TAL1* and *LMO2* both participate in the same transcriptional complex,[27] activation of these genes may both lead to a highly similar expression profile. Combined analysis of *TAL1*- and *LMO2*-rearranged patients revealed significant and differentially expressed probesets that, as expected, almost entirely overlapped with the gene signature obtained for the *TAL1* subgroup only.

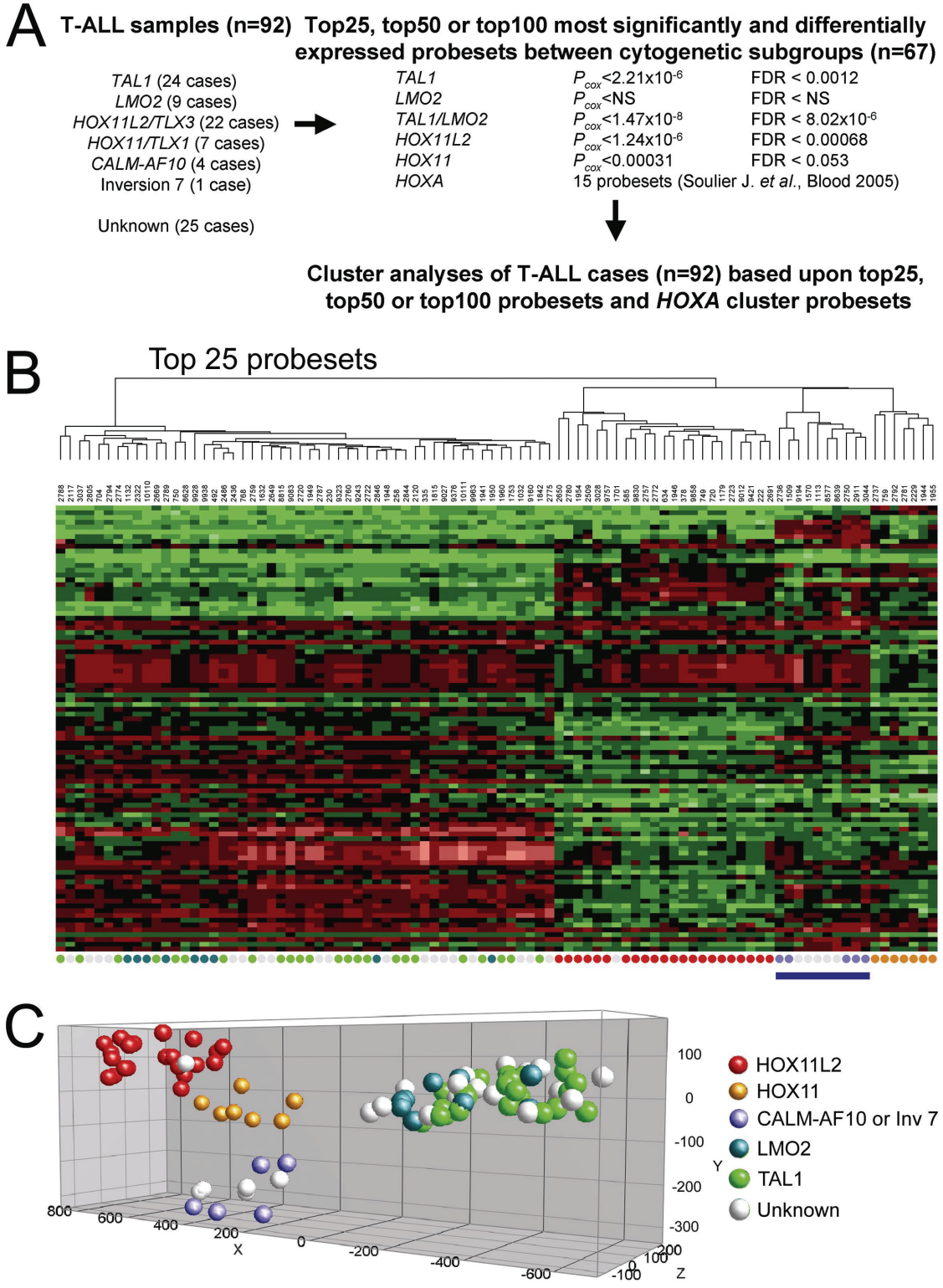

Figure 1. Gene expression profiles of 92 T-ALL patients (for colour figure see page 174)
(A) Differentially expressed genes among the major molecular-cytogenetic T-ALL subgroups (TAL1, LMO2, HOXA, HOX11/TLX1, and HOX11L2/TLX3; n=67). The significance level (Wilcoxon p value) and FDR corrected p value for the top 100 genes in each T-ALL subgroup is indicated. TAL1, HOX11, and HOX11L2 subgroups show significant differentially expressed probesets. (B) Cluster analysis of 92 patients with T-ALL (67 with known, 25 with unknown) based upon the top 25 most significant probesets for the TAL1, TAL1/LMO2, HOX11, and HOX11L2 subgroups combined with 15 HOXA probesets as previously described.[8] (C) Principal component analyses shows clustering of the patients with unknown T-ALL along the molecular-cytogenetic known patients: 1HOX11L2-like, 19 TAL1/LMO2-like, and 5 HOXA-like patients.

Next, we tried to cluster all 92 patients with T-ALL. Cluster analysis was performed based upon the top 25, 50, or 100 most significant probesets for the *TAL1, TAL1/LMO2, HOX11,* and *HOX11L2* subgroups combined with 15 *HOXA* probesets identified by Soulier et al[8] (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article; Figure 1A,B). Cluster and principal component analyses (PCAs) led to a stable clustering of patients with unknown T-ALL with specific molecular-cytogenetic subgroups (Figure 1B,C; Figure S1; Table S2): one patient clustered with *HOX11L2*-rearranged patients, and this patient uniquely highly expressed the *HOX11L2* homologous gene *HOX11L1* (data not shown). A total of 19 unknown patients tightly clustered with *TAL1*- or *LMO2*-rearranged patients. FISH analysis (not shown) revealed *TAL1* and/or *LMO2* homologous rearrangements to the *TCRβ* or *TCRαδ* loci in 5 of these 19 patients (i.e. *TAL2* [1 patient]; *LMO1* [1 patient]; *TAL2/LMO1* [1 patient]; and *cMYC* [2 patients] in line with karyotypic data. Another 5 unknown T-ALL samples formed a separate cluster with the 5 patients with *HOXA*-type T-ALL (Figure 1B,C; Table S2), and will be denoted as *HOXA*-like samples.

New recurrent deletion, del(9)(q34.11q34.13), in *HOXA*-like T-ALL samples

To identify new chromosomal abnormalities in the 5 *HOXA*-like patients, we screened these patients using oligonucleotide array-CGH. A one-copy loss of an approximately 3-Mb region involving chromosomal band 9q34.11 to 9q34.13 was identified in 3 of 5 *HOXA*-like patients (patient nos. 126, 125, and 120; Figure 2A-C). Detailed analysis of the centromeric breakpoints in these 3 patients revealed a breakpoint within or in the vicinity of the *SET* gene. The *PKN3* gene just telomeric to *SET* was consistently lost in all 3 patients (Figure 2A-C). The telomeric breakpoint was located in the *NUP214/CAN* gene in 2 patients (nos. 125 and 126; Figure 2A-C), whereas the telomeric breakpoint of patient no. 120 seemed situated in the *ABL1* oncogene (Figure 2B,C).

The presence of the del(9)(q34.11q34.13) in patient nos. 125 and 126 was confirmed by FISH using the LSI *BCR-ABL* ES translocation probe, resulting in a single-copy loss of the *ABL1* gene (Figure 2D). Strikingly, FISH analysis on patient no. 120 also revealed an identical monoallelic loss of *ABL1* in all leukemic cells. However, an episomal *NUP214-ABL1* amplification[6] was detected in a leukemic subclone comprising approximately 5% of the total leukemic cell population (Figure 2E). Subsequent FISH analysis confirmed that the episomal *NUP214-ABL1* amplification as well as the del(9)(q34.11q34.13) were both present in this subclone (data not shown). The combined presence of a clonal del(9)(q34.11q34.13) in combination with an episomal *NUP214-ABL1* amplification in a leukemic sub-clone in this patient explains why the telomeric breakpoint of the del(9)(q34.11q34.13) seemed situated in the *ABL1* gene according to the array-CGH data. Additional FISH screening of the remaining 87 patients with T-ALL did not reveal other patients with this same deletion.

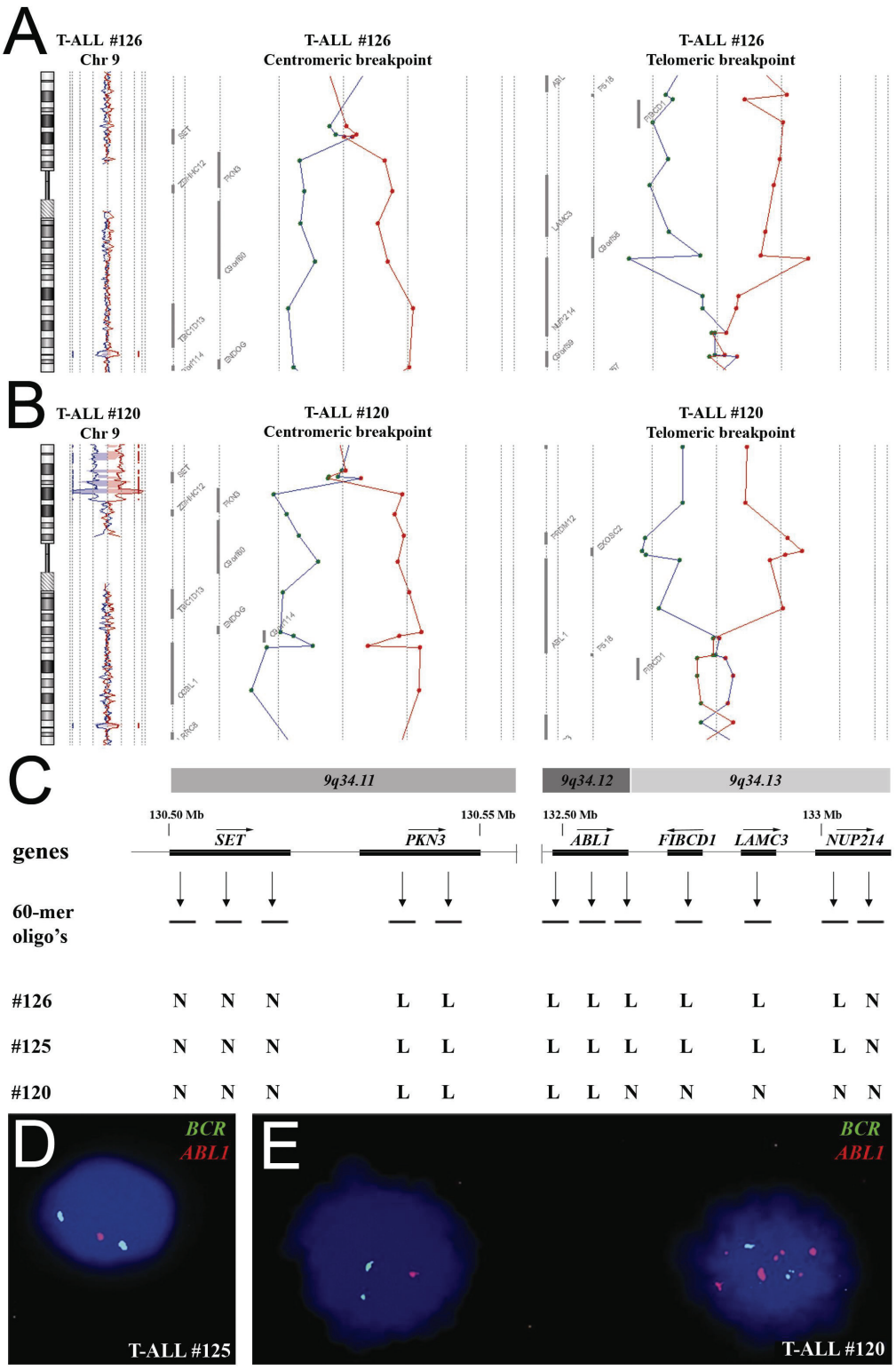

Figure 2. Submicroscopic del(9)(q34.11q34.13) in T-ALL (for colour figure see page 175)
(A) Chromosome 9 ideogram and corresponding oligo array-CGH plots of test DNA–control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for patient no. 126. Detailed analyses of the centromeric and telomeric breakpoints show involvement of SET and NUP214. (B) Similar array-CGH plot for patient no. 120. Centromeric and telomeric breakpoints show involvement of SET and ABL1. (C) Overview of oligoarray-CGH results in the potential breakpoint regions for 3 patients with T-ALL with del(9)(q34.11q34.13). The 60-mer oligonucleotide probes present on the arrayCGH slide and located in the telomeric and centromeric breakpoint regions, as well as the specific genes located in this region with their transcription direction, are shown. Abbreviations: N; normal, L; loss. Dual-color FISH analysis of patient no. 125 (D) and no. 120 (E) using the LSI BCR-ABL ES translocation probe.

SET-NUP214 fusion in del(9)(q34.11q34.13)$^+$ patients

Subsequent RT-PCR analysis to amplify a potential *SET-NUP214* fusion product using a *SET* forward primer (exon 7) in combination with a *NUP214* reverse primer (exon 20) revealed an *SET-NUP214* fusion product in all 3 del(9)(q34.11q34.13)$^+$ patients with T-ALL that we also identified in the T-ALL cell line LOUCY[28] (Figure 3A). A similar *SET-NUP214* fusion product due to the reciprocal chromosomal translocation t(9;9)(q34;q34) has been described previously for a patient with an acute undifferentiated leukemia (AUL) by Von Lindern et al.[29] Material from this patient with AUL was still available, and RT-PCR analysis revealed a *SET-NUP214* fusion product in this patient (Figure 3A). Sequence analyses of the *SET-NUP214* PCR products confirmed that these 3 patients with T-ALL and the patient with AUL,[29] as well as the cell line LOUCY, all had an identical fusion product fusing *SET* at exon 7 with the *NUP214* gene at exon 18 (Figure 3B). Additional oligonucleotide array-CGH analysis further confirmed that the *SET-NUP214* fusion in the LOUCY cell line was indeed due to the presence of a del(9)(q34.11q34.13). This deletion was not present in the patient with AUL, confirming that the *SET-NUP214* fusion was the result of a balanced t(9;9)(q34;q34) in this patient.[29]

RT-PCR analysis also confirmed an episomal *NUP214-ABL1* fusion product present in patient no. 120, as well as in control patient material with an episomal *NUP214-ABL1* amplification (Figure 3A; patient no. 88). Sequence analysis confirmed an in-frame fusion of *NUP214* exon 31 to exon 2 of *ABL1* for patient no. 120.

As expected, the presence of a SET-NUP214 fusion protein was detected by Western blotting in LOUCY (Figures 3C), but was absent in other T-ALL cell lines lacking the del(9)(q34.11q34.13). For all patients described, the breakpoints are situated in the acidic tail of SET and the coiled-coil domain of NUP214, generating an in-frame fusion protein with a molecular weight of approximately 155 kDa (Figure 3D).

Clinical and genetic patient characteristics (i.e. *NOTCH1* mutation status and additional aberrations detected by array-CGH) of the *SET-NUP214*$^+$ patients with T-ALL and the LOUCY cell line are summarized in Table 1.

Elevated HOXA levels in SET-NUP214$^+$ patients

To confirm the clustering of these 3 del(9)(q34.11;q34.13)$^+$ patients within the *HOXA* cluster based upon the most significant and differentially expressed probesets between the major T-ALL subgroups, we analyzed the expression of the *HOXA* gene cluster using RQ-PCR. As a control, we also determined the expression levels for these genes in various other patient samples representing other T-ALL subgroups. As described previously,[8,10,18,19] *MLL*-rearranged cases (n=2), *CALM-AF10*$^+$ cases (n=4), and a patient with an inv(7)(p15q34) mutation all highly expressed most genes from the *HOXA* cluster in contrast to *TAL1-, LMO2-, HOX11L2-,* or *HOX11*-rearranged patients (p<0.01; Figure 4A,B; only results for *HOXA9* are shown). All 3 *SET-NUP214*$^+$ patients with T-ALL as well as the LOUCY cell line also highly expressed the *HOXA* cluster of genes. Other T-ALL cell lines, including MOLT13, SKW3, HPB-ALL, HSB2, and PEER, did not express the *HOXA* gene cluster. Although most *HOXA* genes were highly expressed in LOUCY, in the patient with AUL and in the *SET-NUP214*$^+$ patients with T-ALL, expression of *HOXA11* and *HOXA13* was virtually absent.

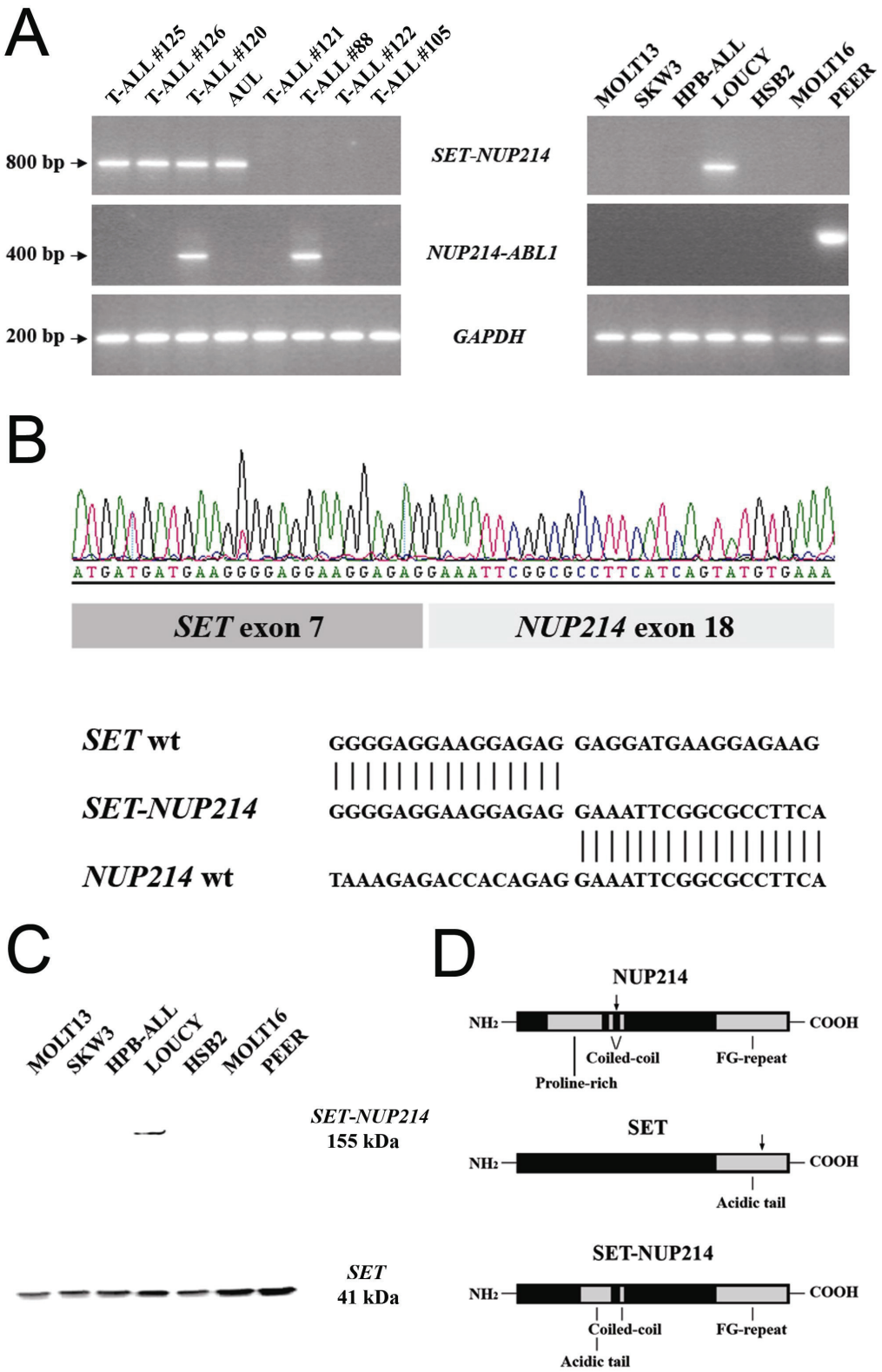

Figure 3. ***SET-NUP214*** **fusion transcript in T-ALL** (for colour figure see page 176)
RT-PCR analysis using SET- and NUP214-specific primers and GAPDH primers as internal control, reveals a specific SET-NUP214 fusion gene in T-ALL patient nos. 125, 126, and 120; the patient with AUL; and the T-ALL cell line LOUCY. NUP214-ABL1 fusion was detected in patient nos. 120 and no. 88 and in the T-ALL cell line PEER (B) Sequence analysis confirmed an identical fusion between exon 7 of SET and exon 18 of NUP214 in all SET-NUP214^{+} patients with T-ALL, the patient with AUL, and the LOUCY cell line. (C) Western blot analysis of T-ALL cell lines revealed a SET-NUP214 fusion in the cell line LOUCY. (D) At the protein level, the breakpoints are situated in the acidic tail of SET and the coiled-coil domain of NUP214.

Table 1. Characteristics of 3 patients with T-ALL and the T-ALL cell line LOUCY with the *SET-NUP214* fusion transcript

patient no.	Sex	Age y	WBC x 10^9/L	Blast %	Immune Phenotype*	CDKN2A†	*NOTCH1* mutation	NUP214-ABL1	Other chromosomal abnormalities by array-CGH	Relapse CCR, mo
126	F	15.3	213	98	Mature	+/+	L1601Q	-	None	CCR,83$^+$
125	F	10.6	142	94	Mature	+/+	L1601P	-	del(16)(p13.13)	CCR,83$^+$
120	F	17.1	15	93	Cortical	-/-	N1683D, Q2460* (stop)	+	del(9)(p21.2), monosomy 21	CCR,37$^+$
LOUCY	F	38	NA	NA	Mature	+/-	ND	-	del(5)(q14.3q31.1) del(5)(q33.1) del(6)(q16.4) del(9)(p21.2p21.3) del(12)(p13.1p13.3) dup(13)(q31.3) del(16)(p12.3)	NA

WBC indicates white blood cells; CCR, continued complete remission; ND, none detected; and NA, not available.
** According to the European Group of Immunological Characterisation of Leukemia (EGIL) classification.*
† Status of the CDKN2A locus: +/+, no deletion; +/-, hemizygous deletion; -/-, homozygous deletion.

In addition, the expression of the short *HOXA10* isoform, *HOXA10B,* which previously has been exclusively associated with patients with inv(7)(p15q34) T-ALL,[8,20] was also highly expressed in the *SET-NUP214*$^{+}$ patients (Figure 4C).

From the expression microarray data, the most significant and differentially expressed probesets were calculated for the entire *HOXA* cluster. A total of 20 significant and differentially expressed probesets with a FDR rate lower than 5% were obtained for this cluster (Figure 4D). Various of these probesets encoding for *QKI, HOXA5, HOXA9* (2 probesets), *HOXA10* (2 probesets), and *HOXA11* were also previously found to be differentially expressed within *MLL*-rearranged[19] or *CALM-AF10*-rearranged[18] patients with T-ALL or in patients with T-ALL belonging to the *HOXA* subgroup.[8]

SET-NUP214 activates *HOXA* expression, increases cellular proliferation, and inhibits cellular differentiation

To study the role of the *SET-NUP214* fusion transcript in the pathogenesis of T-cell leukemia and its contribution to the activation of the *HOXA* gene cluster, *SET* and *SET-NUP214* expression were specifically down-regulated in the LOUCY cell line by electroporation of *SET*-specific siRNAs (Figure 5). Protein expression of SET and SET-NUP214 was specifically reduced using a *SET* siRNA directed against exon 5, whereas only SET but not SET-NUP214 was down-regulated using a *SET* siRNA directed against exon 8 (Figure 5A). *SET* and/or *SET-NUP214* mRNA expression levels were specifically targeted, and this effect was sustained for more than 7 days following transfection of *SET* siRNAs (Figure 5B). Specific down-regulation of both SET and SET-NUP214 resulted in significant reduction in the expression levels of the *HOXA* gene cluster, while knockdown of SET but not SET-NUP214 had no effect (Figure 5C). This confirms that SET-NUP214, or the combination of SET and SET-NUP214, specifically up-regulates the expression of *HOXA* genes. Knockdown of SET-NUP214 also reduced cellular proliferation (Figure 5D,E), but the percentage of apoptotic cells did not change over time following inhibition of SET-NUP214 (Figure 5F). In addition, SET-NUP214 down-regulation resulted in the up-regulation of both TCRγδ and membrane CD3 expression in LOUCY (Figure 5G), indicating that repression of SET-NUP214 enforces differentiation. Down-regulation of SET by anti-SET siRNA exon 8 had no effect (Figure 5G). The effects of *SET-NUP214* knockdown (*HOXA* inhibition, mild reduced cell proliferation, and induction of differentiation) were confirmed using a second *SET* siRNA directed against exon 3 (Figure S3).

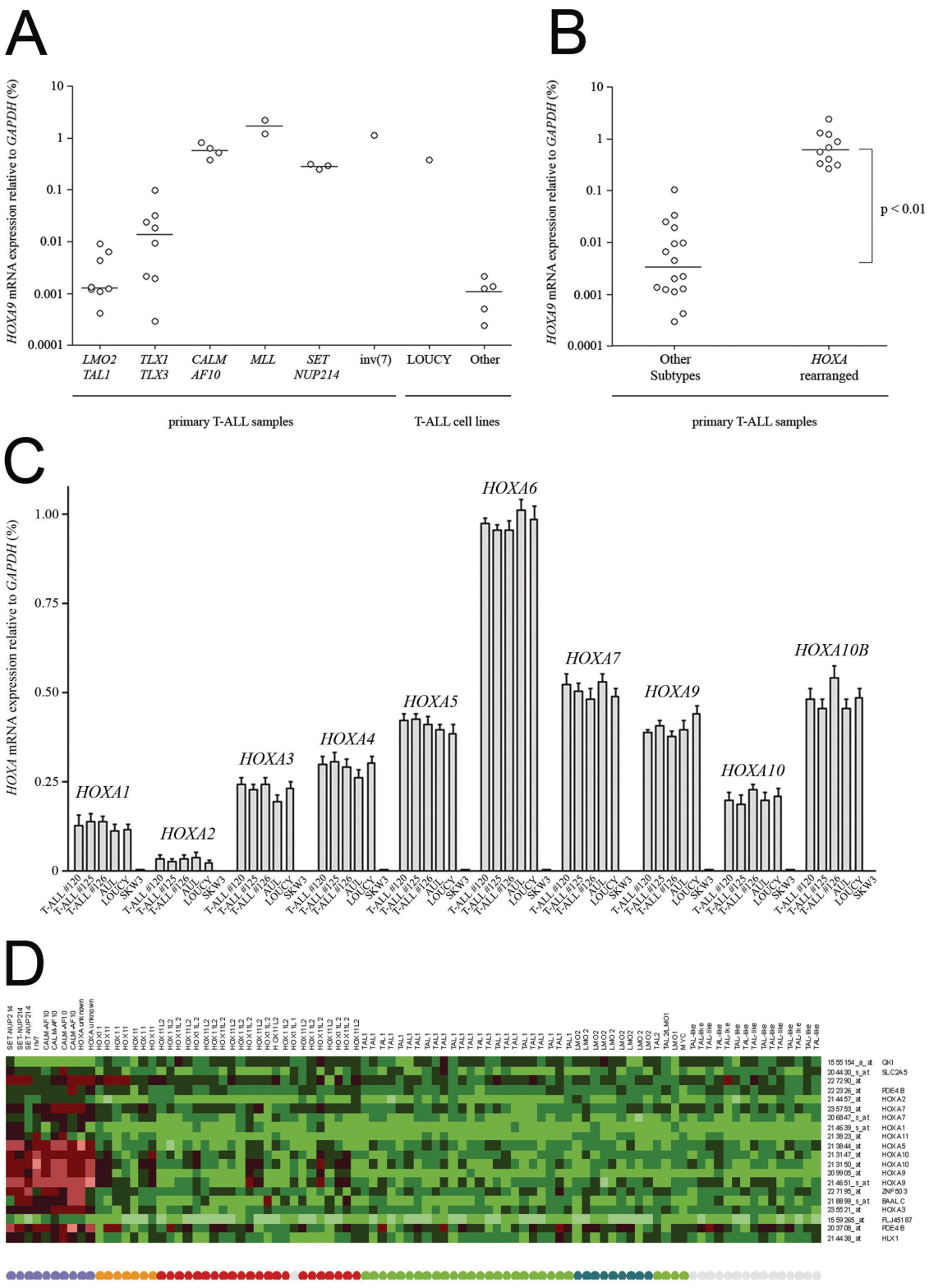

Figure 4. ***HOXA* activation in *SET-NUP214*$^{+}$ T-ALL** (for colour figure see page 177)
(A) Relative HOXA9 expression levels by RQ-PCR for MLL-rearranged, CALM-AF10^{+}, inv(7)(p15q34), TAL1-, LMO2-, HOX11L2-, or HOX11-rearranged patients and T-ALL cell lines including LOUCY, MOLT13, SKW3, HPB-ALL, HSB2, and PEER. (B) Comparison of HOXA9 expression levels between the HOXA T-ALL subgroup (MLL, CALM-AF10, inv(7)(p15q34), and SET-NUP214; n=10) and other T-ALL subgroups (TAL1, LMO2, HOX11L2, or HOX11). The horizontal lines represent the medians. (C) Relative expression levels of HOXA genes by RQ-PCR for the 3 SET-NUP214^{+} patients with T-ALL, the LOUCY cell line, the patient with AUL, and SKW3. (D) Heatmap of the 20 significant and differentially expressed probesets with a false discovery rate (FDR) lower than 5% for the HOXA cluster compared with the other patients with T-ALL.

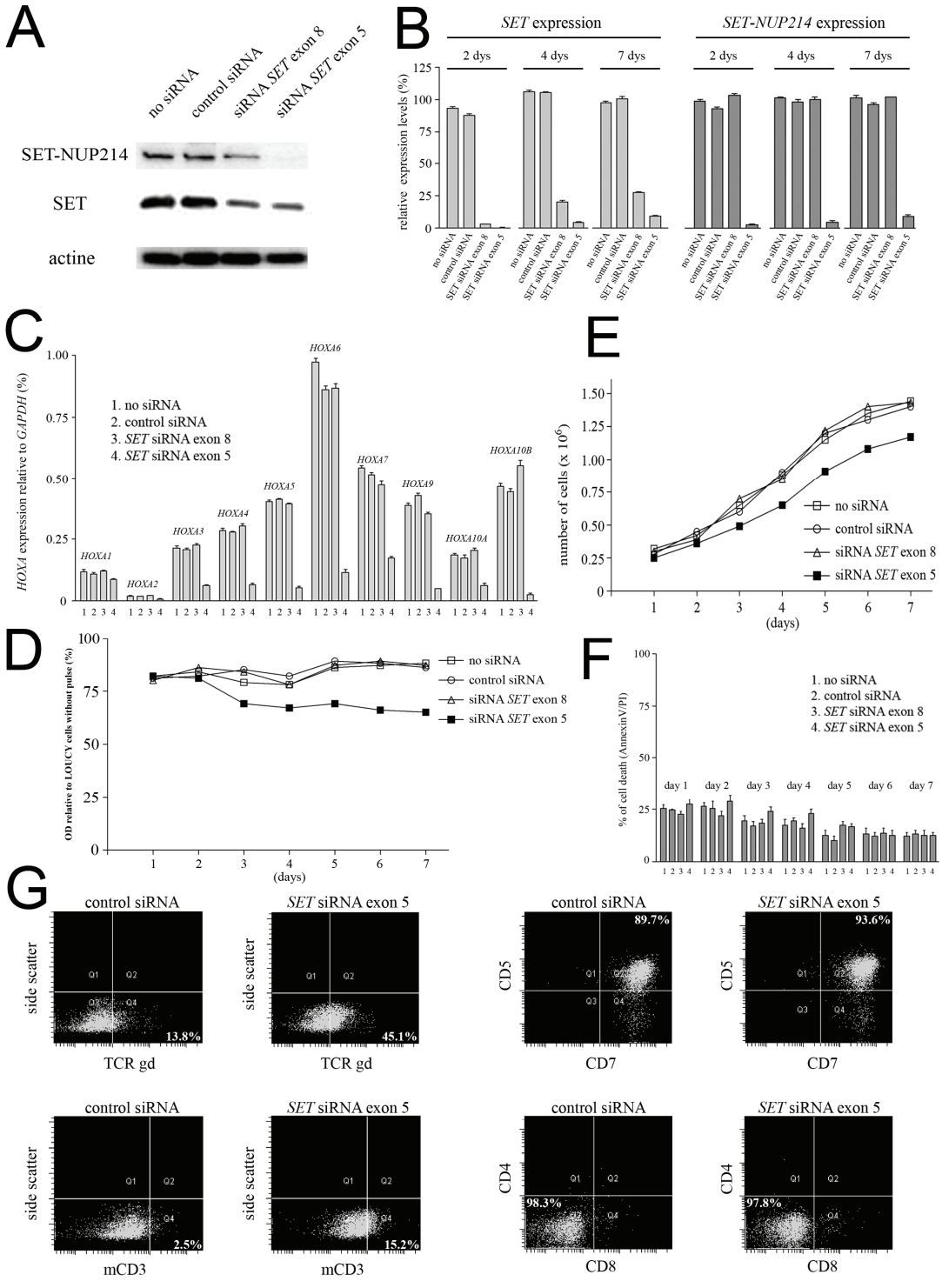

Figure 5. siRNA knockdown of SET and *SET-NUP214* in the LOUCY T-ALL cell line

(A) SET expression using Western blot analysis 4 days after electroporation with the following conditions: no siRNA, control siRNA, siRNA SET exon 8, or siRNA SET exon 5. (B) Relative SET and SET-NUP214 expression by RQ-PCR after 2, 4, and 7 days for conditions as mentioned in panel A. (C) Relative expression of all members of the HOXA clusters by RQ-PCR (except for HOXA11 and HOXA13) after 4 days for conditions as mentioned in panel A. (D) Optical density (OD) values relative to control cells without pulse after 7 days for conditions as mentioned in panel A. (E) Total cell numbers after 7 days for conditions as mentioned in panel A. (F) Percentage of cell death relative to control cells without pulse after 7 days for conditions as mentioned in panel A. Error bars visualize the standard error of the mean (SEM). (G) FACS analysis 6 days after electroporation with either no siRNA, siRNA SET exon 8, or siRNA SET exon 5 for TCRγδ and membrane CD3. The percentage of cells positive for TCRγδ or mCD3 expression are visualized in quadrant 4.

SET-NUP214 directly activated *HOXA* expression, possibly by recruitment of DOT1L

Our siRNA-mediated knockdown experiments indicated that SET-NUP214 regulates the transcription of the *HOXA* gene cluster. To investigate whether this activation was caused by direct interaction of SET-NUP214 with *HOXA* promoter sequences, ChIP analyses with the LOUCY cell line and the negative control cell line SKW3 were performed. No enrichment of *HOXA* (*HOXA1, HOXA3, HOXA9, HOXA10,* and *HOXA11)* promoter sequences was detected in NUP214 immunoprecipitates obtained for SKW3 control cells (Figure 6A). For LOUCY cells, *HOXA9* and *HOXA10* promoter sequences were enriched in NUP214 immunoprecipitates, but not *HOXA1 , HOXA3,* and *HOXA11* promoter sequences, indicating that SET-NUP214 may only bind to specific members of the *HOXA* cluster (Figure 6A). Enrichment of *HOXA9* and *HOXA10* promoter sequences in the ChIP analysis could be completely reversed using *SET* siRNA molecules directed against exon 5.

Additional ChIP analysis using antiacetyl histone H3 (Figure S2)- and antidimethyl histone H3K79 (Figure 6A)-specific antibodies revealed histone H3 acetylation and histone H3K79 methylation of *HOXA1, HOXA3, HOXA9, HOXA10* and *HOXA11* promoters in the LOUCY cell line, which was absent in SKW3. This further strengthens the idea that binding of SET-NUP214 as a specific transcriptional cofactor for some *HOXA* gene members may result in an open chromatine structure of the entire *HOXA* cluster. Upon *SET-NUP214* inactivation, the histone H3K79 methylation was partially lost (Figure 6A), indicating that sustained histone H3K79 methylation of the HOXA locus depended on the presence of SET-NUP214. The level off histone H3 acetylation of the *HOXA* locus remained unaltered within the time frame of the experiment (Figure S2). As SET normally associates with the *HOXA* gene cluster[30] and has an inhibitory role on gene transcription as part of the INHAT complex,[31] we investigated whether SET-NUP214 may substitute for SET in this complex, rendering this complex inactive. However, IP experiments failed to demonstrate a direct interaction between SET-NUP214 and components of the INHAT complex (i.e. SET and PP32; Figure 6B).

CALM-AF10, MLL-AF10, MLL-ENL, and *MLL-AF4* fusion proteins have been shown to recruit the methyltransferase DOT1L, leading to aberrant methylation of histone H3 at lysine 79, thereby facilitating transcriptional activation of the *HOXA* gene cluster.[32-35] For CALM-AF10 and MLL-AF10, this interaction with DOT1L depends on the octapeptide and leucine zipper domain (OM-LZ region) in the AF10 part of these fusion proteins.[32,33] With respect to the histone H3-K79 methylation of the *HOXA* locus in our SET-NUP214^{+} patients, we could demonstrate an interaction between SET-NUP214 and DOT1L in vivo (Figure 6C). However, this interaction seemed independent of the presence of AF10, since an interaction between SET-NUP24 and AF10 could not be demonstrated in LOUCY (Figure 6D). Reciprocal DOT1L IP experiments to study AF10 involvement could not be performed, as suitable DOT1L antibodies for IP experiments are not available. Whether the interaction between DOT1L and SET-NUP214 is a direct interaction or depends on the participation of additional proteins remains to be established. As shown by others,[36,37] we could confirm an in vivo interaction between SET-NUP214 and CRM1 (Figure 6C), which normally binds to the FG-repeat of wild-type NUP214 as part of the nuclear pore complex.[36]

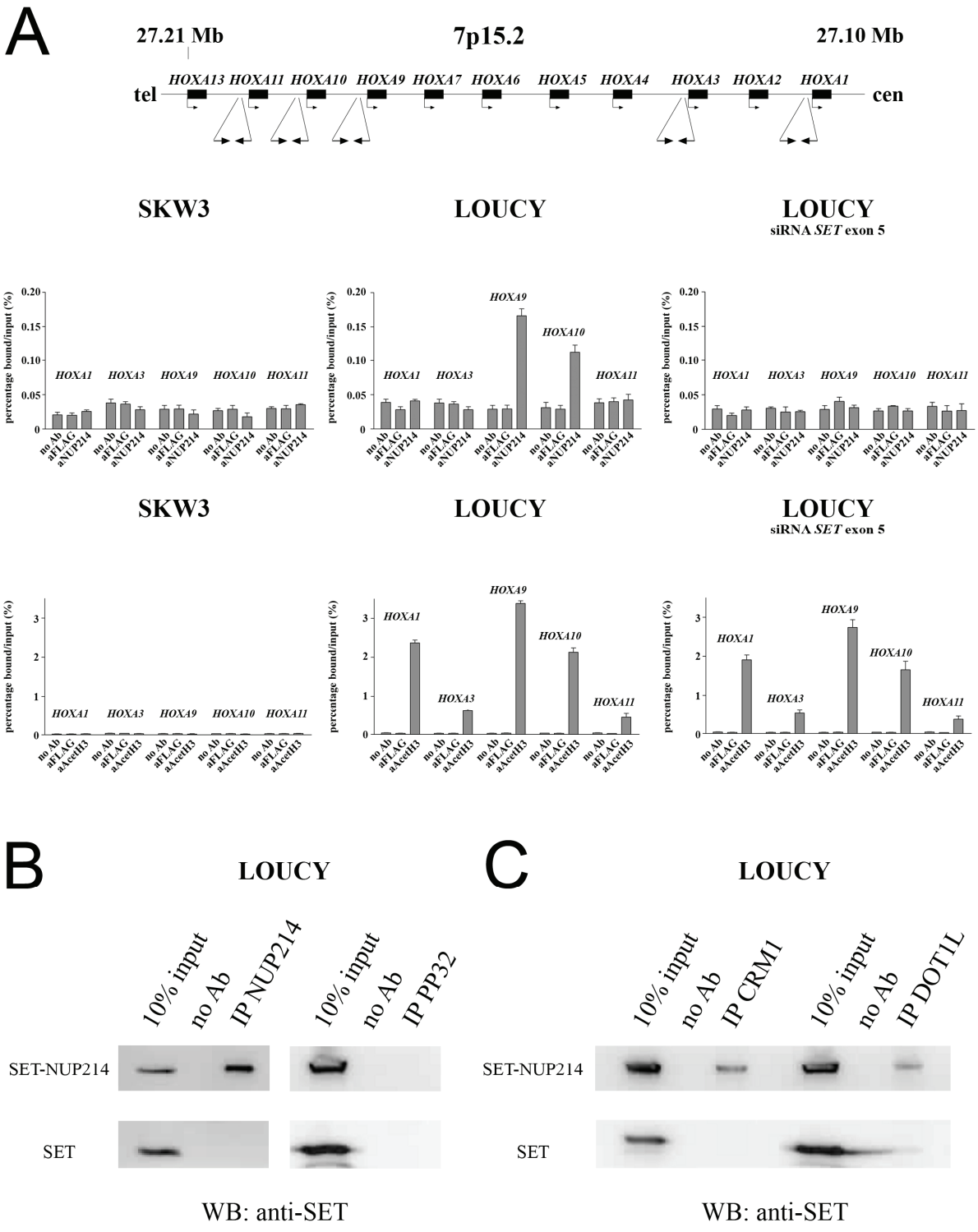

Figure 6. ChIP and coIP analysis of T-ALL cell lines LOUCY and SKW3
(A) ChIP analysis of SKW3, LOUCY, and LOUCY 4 days after electroporation with siRNA SET exon 5, for promoter sequences of HOXA1, HOXA3, HOXA9, HOXA10, and HOXA11. The amount of bound DNA was calculated relative to the 5% input DNA in anti-NUP214 and anti-acetyl histone H3 immunoprecipitates, whereas no antibody and anti-FLAG immunoprecipitates were used as negative control. Error bars represent SEM. (B) Western blot analysis of NUP214 and PP32 immunoprecipitates of the cell line LOUCY using anti-SET. (C) Similar Western blot analysis as in panel B for CRM1 and DOT1L immunoprecipitates in LOUCY. (D) Western blot analysis of NUP214 immunoprecipitates of the cell line LOUCY using anti-AF10.

DISCUSSION

Gene expression profiling studies in T-ALL have shown that patients with a *CALM-AF10* translocation, an *MLL* rearrangement, or an inv(7)(p15q34) mutation share a gene expression signature characterized by elevated expression levels of *HOXA* genes. Cluster analysis of 25 patients with T-ALL lacking known cytogenetic abnormalities with 67 cytogenetically well-characterized patients led to the identification of 5 patients with unknown disease that clustered with *HOXA*-activated samples having *CALM-AF10* translocations or an inv(7)(p15q34) mutation.

Subsequent array-CGH analysis revealed an identical interstitial deletion on chromosome 9 [i.e. the del(9)(q34.11q34.13)], in 3 of 5 patients as a novel chromosomal aberration in pediatric T-ALL that we also identified in the T-ALL cell line LOUCY. This deletion gives rise to a similar *SET-NUP214* fusion gene in all cases, and was identical to a SET-NUP214 fusion as described 15 years ago for a single acute undifferentiated leukemia patient with a reciprocal translocation t(9;9)(q34; q34),[29,38] and most recently for a single patient with acute myeloid leukemia.[39]

We studied the role of SET-NUP214 in the pathogenesis of T-ALL by siRNA-mediated knockdown of SET-NUP214 in the LOUCY cell line. Down-regulation of SET-NUP214 reduced *HOXA* expression levels, indicating that SET-NUP214 could function as a transcriptional regulator of the *HOXA* gene cluster. Our ChIP data in fact provide evidence that SET-NUP214 directly interacts with the promotor regions of specific *HOXA* members itself, especially with *HOXA9* and *HOXA10,* and therefore may function as a transcriptional coactivator. SET-NUP214 does not bind to promotor sequences of *HOXA1, HOXA3, HOXA11,* and possibly others despite their SET-NUP214 dependency for transcriptional activation in LOUCY. Binding of SET-NUP214 to *HOXA9* and *HOXA10* promoter regions presumably may lead to an open chromatin structure and transcriptional activation of the entire *HOXA* cluster.

Based upon the ChIP analyses using antiacetylated histone H3 and antidimethyl histone H3-K79 before and after siRNA-mediated SET-NUP214 knockdown, we hypothesize that binding of SET-NUP214 at the *HOXA* promoters may facilitate the recruitment of the H3-K79 methyltransferase DOT1L, resulting in local H3-K79 methylation as a first direct chromatine modification effect. This was supported by our data, in which histone H3-K79 methylation of the *HOXA* locus was partially lost upon inhibition of SET-NUP214 within the (limited) time frame of the experiment. This modification may further trigger the acquisition of other "open state" chromatin modifications, such as H3-acetylation possibly enabling the recruitment of other transcriptional cofactors and transcriptional activation of various *HOXA* gene members.

For MLL-AF10 and CALM-AF10 fusion proteins, it has already been demonstrated that the oncogenicity of these proteins depends on binding of DOT1L to the OM-LZ region of AF10.[32,33] MLL-AF10 and CALM-AF10 both bind to the promoter regions of the *HOXA* gene cluster, and it was shown that recruitment of DOT1L results in aberrant methylation of Lys79 in histone H3 and transcriptional activation of especially *HOXA9* for MLL-AF10[33] and *HOXA5* for CALM-AF10.[32] In addition, recent reports also show interaction of DOT1L with the fusion proteins MLL-ENL and MLL-

AF4.[34,35] Our data suggest that *HOXA9* may also represent a bona fide target of the CALM-AF10 fusion protein, as *HOXA9* is highly expressed in CALM-AF10$^+$ T-ALL cells (Dik et al[18] and this study). We propose a similar mechanism for SET-NUP214 in the activation of *HOXA* genes, and *HOXA9*/*HOXA10* in particular, as we could demonstrate binding of DOT1L to the SET-NUP214 fusion protein. An OM-LZ region as present in AF10[33] was not identified in SET-NUP214, and therefore another OM-LZ–like region in SET-NUP214 or an indirect interaction between SET-NUP214 and DOT1L may be facilitated by other SET-NUP214-interacting proteins. In this respect, we confirmed that CRM1 also binds to SET-NUP214,[37] possibly to the FG repeats as retained in this fusion protein.

SET-NUP214 is highly similar to the DEK-NUP214 fusion as previously identified in t(6;9)(p23;q34)$^+$ patients with AML.[40] As DEK-NUP214 AML samples also have an activated *HOX*A gene signature (P.J. Valk, oral communication, January 2008), DEK-NUP214 may function in a similar fashion compared with SET-NUP214 by binding to the promotor regions of specific *HOXA* gene members in t(6;9)(p23;q34)$^+$ patients with AML.

SiRNA knockdown experiments in LOUCY led to complete absence of SET-NUP214 and down-regulation of *HOXA* expression levels that sustained for more than 7 days. Ablation of SET-NUP214 had a mild effect on cellular proliferation without inducing apparent apoptosis in this timeframe. In fact, inhibition of SET-NUP214 may have resulted in cellular differentiation and promoted mCD3 and TCRγδ expression. Our results are in agreement with previous data by others in which overexpression of SET-NUP214 inhibits differentiation in vitro[41] as well as in vivo.[42]

During normal T-cell development, *HOXA* expression (*HOXA7, HOXA9, HOXA10)* is restricted to the earliest stages of differentiation.[43,44] We therefore propose that SET-NUP214 will sustain *HOXA* gene expression and therefore impair T-cell differentiation. This differentiation arrest may encourage the acquisition of additional genetic hits, eventually leading toward the development of T-cell leukemia. In mouse studies, overexpression of *Hoxa10* inhibits both myeloid and lymphoid cell differentiation,[45] whereas overexpression of *Hoxa9* results in defective T-cell development.[46]

T-cell leukemia depends on multistep pathogenic events.[3-5] Cooperative genetic abnormalities affecting cell cycle and proliferation, differentiation, and survival initiate leukemic transformation of thymocytes. Since *SET-NUP214* fails to cause T-ALL in transgenic mice,[42,47] additional mutations will be required for the induction of T-cell leukemia. We identified a number of cooperative aberrations in the *SET-NUP214*$^+$ T-ALL samples. *NOTCH1* mutations, generally present in about 50% of T-ALL,[7] were found in all 3 *SET-NUP214*$^+$ T-ALL samples. Besides the *SET-NUP214* fusion (differentiation arrest) and *NOTCH1* mutations, patient no. 120 also showed a homozygous *CDKN2A/CDKN2B* deletion (cell-cycle defect) and an episomal *NUP214-ABL1* amplification (proliferation and survival), showing the multiple molecular pathways that are involved in the pathogenesis of T-ALL.[4] It is remarkable that in this patient, 2 different genetic rearrangements (*SET-NUP214* and *NUP214-ABL1)* target the same gene (*NUP214)* in a single leukemic cell. The SET-NUP214 fusion was present as a clonal genetic rearrangement present in all leukemic cells, whereas NUP214-ABL was only present in a leukemic subclone. So SET-NUP214 probably acts

as a primary oncogenic event, whereas NUP214-ABL1 rather functions as a further dedifferentiating event in T-ALL. In patient no. 125 and the LOUCY cell line, terminal deletions of chromosome 16 were identified (Table 1). Because these 16p deletions were not previously identified as a recurrent abnormality in T-ALL,[48] it is likely that they cooperate in *SET-NUP214*–mediated leukemogenesis. Nevertheless, the target genes of this aberration remain to be identified.

In conclusion, we identified *SET-NUP214* as a novel recurrent fusion gene in T-cell leukemia. Our experiments show that *SET-NUP214* may contribute to T-ALL pathogenesis by inhibition of T-cell maturation through the transcriptional activation of the *HOXA* genes.

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Prognostic significance of molecular-cytogenetic abnormalities in pediatric T-ALL is not explained by immunophenotypic differences

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ABSTRACT

Pediatric T-cell acute lymphoblastic leukemia (T-ALL) is characterized by chromosomal rearrangements possibly enforcing arrest at specific development stages. We studied the relationship between molecular-cytogenetic abnormalities and T-cell development stage to investigate whether arrest at specific stages can explain the prognostic significance of specific abnormalities. We extensively studied 72 pediatric T-ALL cases for genetic abnormalities and expression of transcription factors, *NOTCH1* mutations and expression of specific CD markers. *HOX11* cases were CD1 positive consistent with a cortical stage, but as 4/5 cases lacked cytoplasmatic-β expression, developmental arrest may precede β-selection. *HOX11L2* was especially confined to immature and pre-αβ developmental stages, but 3/17 *HOX11L2* mature cases were restricted to the γδ-lineage. *TAL1* rearrangements were restricted to the αβ-lineage with most cases being TCR-αβ positive. *NOTCH1* mutations were present in all molecular-cytogenetic subgroups without restriction to a specific developmental stage. *CALM-AF10* was associated with early relapse. *TAL1* or *HOX11L2* rearrangements were associated with trends to good and poor outcomes, respectively. Also cases with high vs low *TAL1* expression levels demonstrated a trend toward good outcome. Most cases with lower *TAL1* levels were *HOX11L2* or *CALM-AF10* positive. *NOTCH1* mutations did not predict for outcome. Classification into T-cell developmental subgroups was not predictive for outcome.

INTRODUCTION

Treatment of pediatric acute lymphoblastic leukemia (ALL), has greatly improved over the last decades, leading to cure in approximately 80-85%. T-cell ALL (T-ALL) accounts for ~15% of all newly diagnosed pediatric ALL cases, and is clinically regarded as a high-risk disease with a relapse rate of about 30% (reviewed in Pui et al.[1] and Thiel et al.[2]).

In about half of the pediatric T-ALL patients recurrent chromosomal translocations are being found (reviewed in De Keersmaecker et al.,[3] Raimondi[4] and Rubnitz and Look[5]). For most of these abnormalities, the T-cell receptor (TCR)-α/δ or TCR-β loci are involved[6] presumably as mistakes during attempted gene segment rearrangements. Other recurrent translocation partners include basic helix-loop-helix (bHLH) genes (*MYC*, *TAL1*, *TAL2, LYL1* and *bHLHB1*), cysteine-rich (LIM-domain containing) genes (*LMO1* and *LMO2*), homeodomain genes (*HOX11/TLX1, HOX11L2/TLX3* or the *HOXA* gene cluster) or, as identified most recently, the *MYB* oncogene.[7] Other translocations leading to the formation of specific fusion genes like *CALM-AF10*,[8] or *MLL* rearrangements have also been described.[4,5] These rearrangements in general occur in a mutually exclusive manner and allows for the identification of specific T-ALL subgroups. However, other recurrent chromosomal abnormalities such as 6q deletions, deletion of 9p21 including the *p15* and *p16* loci, episomal amplification of a *NUP214-ABL1* fusion or *MYB* duplications are shared by various of these T-ALL subgroups.[3,9,10] Activating mutations in *NOTCH1* have been identified in more than 50% of all T-ALL cases that are also shared by various T-ALL subgroups.[11]

The presence of specific molecular-cytogenetic aberrations may predict for outcome in T-ALL. *CALM-AF10* may define a poor prognostic subgroup,[8,12] but the prognostic relevance of *HOX11L2* has proved diverse and may depend on the therapy protocol.[12-17] *HOX11* abnormalities have been associated with a favorable outcome.[14,18-20] The prognostic significance of *NOTCH1* mutations is currently unknown, but in one study mutations were associated with a favorable outcome.[21]

The prognostic significance of these molecular-cytogenetic subgroups may depend on differences in maturation arrest during T-cell development.[22] *CALM-AF10* and *HOX11L2* rearrangements are predominantly found in cases with an early T-cell development along the γδ-lineage, whereas *HOX11* and *TAL1* abnormalities have been associated with the cortical and mature stage along the αβ-lineage of T-cell development.[8,23-25] The European Group for the Immunological Characterization of Leukemias (EGIL)[26] has previously defined four developmental stages in T-ALL for the classification into specific development subgroups depending on the expression of specific cluster of differentiation markers (CD-markers).[27,28] Macintyre and co-workers[23] have developed an alternative classification system that is based upon the TCR status that may better reflect T-cell development as it is currently understood. Alike recognition of specific molecular-cytogenetic subgroups, the recognition of specific T-cell developmental subgroups may also have prognostic relevance. For instance, adult T-ALL cases with a EGIL pro-/pre-T-cell immunophenotype have demonstrated reduced remission induction, early relapse and shortened overall

survival time.[2,29,30] Complete remission (CR) rate was also lower for adult T-ALL with an immature (IM) immunophenotype according to the TCR classification system on the Lala-94 protocol. However, the overall relapse incidence was comparable for all TCR classification subgroups.[31] In children this is less well known, CD1-positive T-ALL have been associated with an excellent outcome,[32,33] and is expressed in most *HOX11*-positive T-ALL patients.[14,34]

In the present study, we have classified pediatric T-ALL patient samples into various immunophenotypic subgroups based upon EGIL[26] or TCR classification criteria.[23] For the various subgroups, the presence of cytogenetic abnormalities as well as the expression of early T-cell transcription factors was investigated. As cytogenetic abnormalities have been associated with outcome, it was investigated whether differentiation status may provide an explanation for the outcome differences between various cytogenetic subgroups.

MATERIALS AND METHODS

Patients

Viably frozen diagnostic bone marrow (n=41) or peripheral blood samples (n=31) from 72 pediatric T-ALL patients, diagnosed between 1991 and 2000, treated according to the Dutch Childhood Oncology Group (DCOG) protocols ALL-7 (n=4), ALL-8 (n=26) or ALL-9 (n=42), were studied.[35,36] Clinical and immunophenotypic data of these patient samples were kindly provided by the DCOG. The median follow-up time for this cohort was 63.5 months. All patient samples were processed as previously described and contained >90% of leukemic blasts.[37] Informed consent was obtained in accordance to the Declaration of Helsinki. Bone marrow biopsies from 28 non-leukemic children (10 without evidence of a hematological malignancy, 2 Burkitt non-Hodgkin's lymphomas, 5 Hodgkin's lymphomas, 5 neuroblastomas, 4 rhabdomyosarcomas and 2 Ewing's sarcomas) were used as a control group to determine *TAL1, LYL1, LMO1* and *LMO2* reference levels. For all patients, the clinical parameters, T-ALL subgroup, *NOTCH1* status, karyotypic results and individual immunophenotypic parameters have been summarized in Supplementary Table 1.

Quantitative real-time RT-PCR

RNA extraction and reverse transcription were performed according to the procedure described previously.[37] Expression levels of *HOX11*, *HOX11L2*, *TAL1, LYL1, LMO1, LMO2* transcripts and *SIL-TAL* and *CALM-AF10* fusion products, were quantified relative to the expression level of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) by real-time RT-PCR in an ABI 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) such as described previously.[12,37,38] The *SIL-TAL1* primers (ENF601, ENR664) and probe (ENP641) for the detection of a *SIL-TAL1* deletion were used as recommended by the Europe Against Cancer Program.[39]

Table 1. Relation between clinical parameters and immunophenotype

	Total n (%)	Sex n M, n F	Median age at Dx (in years) (range)	Median WBC count, 10^9 per litre (range)	Mediastinal involvement n (%)
	72 (100)	51 M, 21 F	6.8 (1.1-16.7)	135 (5-900)	35 (100)
EGIL Classification					
Pro-/pre-T	24 (33)	16 M, 8 F	6.8 (1.1-13.4)	130 (14-590)	11 (31)
Cortical T	30 (42)	23 M, 7 F	7.1 (1.5-15.9)	140 (27-600)	16 (46)
Mature T	18 (25)	12 M, 6 F	7.3 (1.8-16.7)	156.5 (5-900)	8 (23)
TCR Classification					
Immature	26 (36)	18 M, 8 F	6.35 (1.1-15.7)	126.4 (13-530)	14 (40)
PreAB	19 (26)	15 M, 4 F	6.3 (1.8-13.9)	130 (28-600)	7 (20)
TCR GD	5 (7)	3 M, 2 F	5.8 (1.3-15.9)	231 (90-500)	3 (9)
TCR AB	17 (24)	12 M, 5 F	10.4 (2.8-16.7)*	136 (5-900)	8 (23)
Undefined	5 (7)	3 M, 2 F	6.2 (3.8-10.8)	191 (92-347)	2 (6)

Abbreviations: Dx, diagnosis; EGIL, The European Group for the Immunological Characterization of Leukemias; F, female; M, male; n, number; TCR, T-cell receptor; WBC, white blood cell count; Mediastinum, mediastinal involvement; significant associations between clinical and immunophenotypic parameters are indicated with an asterisk.

FISH analysis

Experiments were performed on interphase preparations as described before.[40] Dual-colored FISH experiments to study the presence of the *CALM-AF10* fusion have been described before.[12] For detection of *LMO1* and *LMO2* rearrangements, BAC probe (BacPac Resources, Oakland, CA, USA) combinations RP11-79E12 and RP11-21N2 or RP1189C11 and RP11580K7 were used, respectively. BAC DNA was nick translated using biotin-16-dUTP/digoxigenin-11-dUTP (Roche, Penzberg, Germany). Patient samples were scored positive when more than 10% of interphase cells demonstrated fusion or split apart signals for 100 counted cells by two independent observers. *MLL-, HOX11-, HOX11L2-* or *TAL1*-translocations, or the *SIL-TAL1* deletion[41] was determined by commercially available FISH kits provided by DakoCytomation (Glostrup, Denmark), hybridized and scored as described by the manufacturer.

Immunophenotypic cytoplasmic TCR-β (Cyt-β) and TCR-αβ or TCR-γδ analyses

Indirect cytoplasmic TCR-β (Cyt-β) staining was performed on acetone-fixed cytospin preparations using the antibody βF1 (BioAdvance, Emerainville, France). Following 15min incubation with the βF1 antibody, a secondary incubation with GαM-FITC was performed for visualization. Cyt-β positivity was scored using fluorescence microscopy by two independent observers. TCR expression on the T-ALL samples was analyzed by standard flowcytometry using antibodies against TCR-αβ (BMA031; Beckman Coulter, Fullerton, CA, USA) and TCR-γδ (11F2; Becton Dickinson, San Jose, CA, USA) in combination with staining for CD3 expression. Measurement and acquisition of data were done on FACScan and FACSCalibur flowcytometers (Becton Dickinson). The central reference laboratory of the DCOG determined other immunophenotypic parameters, following standard procedures and a predefined diagnostic panel of reagents.[42] A sample was considered positive when 25% of the leukemic cells or higher expressed a specific immunophenotypic marker. T-ALL patients were assigned into specific subgroups comparable to the EGIL[26] or the TCR classification[23] systems.

Briefly, patients were assigned to the pro-/pre-T-cell subgroup ($CD7^+$, $CD2^+$ and/or $CD5^+$ and/or $CD8^+$, but $CD1^-$ and $sCD3^-$), the cortical T ($CD1^+$) or the mature T ($sCD3^+/CD1^-$) subgroup in accordance to EGIL criteria. In accordance to the TCR-based classification system, patients were assigned to the IM (Cyt-β^-, $sCD3^-$, TCR-$\alpha\beta^-$ and TCR-$\gamma\delta^-$), the pre-$\alpha\beta$ (Cyt-β^+, $sCD3^-$, TCR-$\alpha\beta^-$ and TCR-$\gamma\delta^-$) or the TCR-$\alpha\beta$ ($sCD3^+$, TCR-$\alpha\beta^+$) or TCR-$\gamma\delta$ subgroups ($sCD3^+$, TCR-$\gamma\delta^+$).

NOTCH1 mutations

NOTCH1 mutation screening was performed according to the procedure as described previously.[11] All samples were directly sequenced.

Statistical analysis

All statistical analyses were performed using SPSS 12.0. software. The correlation between the EGIL and TCR classification to assign T-ALL patients to various subgroups was tested using the Spearman's correlation test. Kaplan–Meier curves were constructed, and the log-rank *p*-values were calculated. For the calculation of the disease-free survival (DFS), events were defined as relapse or non-response to induction therapy. The Mann-Whitney *U*-test (MWU) was used to analyze differences in age, WBC and gene expression levels between subgroups. Distribution of positive cases among EGIL or TCR classification subgroups was tested using the Fisher's exact test.

RESULTS

To study the relation between recurrent molecular-cytogenetic abnormalities, T-cell development stage and outcome, patients were assigned into T-cell development subgroups as developed by EGIL[26] and by the Macintyre group[31] (TCR classification). In accordance to EGIL criteria, T-ALL patients were assigned to the pro-/pre-T subgroup (n=24), the cortical subgroup (n=30) and the mature T-cell subgroup (n=18). For 67 patients, the Cyt-β expression status was successfully determined allowing classification according to the TCR classification criteria: 26 patients were assigned to the IM subgroup, 19 patients to the pre-αβ subgroup and 22 patients to the mature subgroup with 5 cases expressing TCR-γδ and 17 cases expressing TCR-αβ (Table 1). The IM T-ALL cases were not further differentiated into IM0, IMδ, IMγ and IMβ subgroups[31] for this study.

Clinical parameters of EGIL and TCR subgroups

Clinical characteristics in relation to EGIL or TCR classification subgroups are depicted in Table 1. For these, no significant relationships were observed with gender, white blood cell count (WBC) or mediastinal involvement, except for age. The median age for TCR-αβ-positive patients (10.4 years) was significantly higher compared to other patients (6.3 years; p=0.02).

Using both classification systems, more than half of the patient samples were assigned to comparable developmental stages, that is, pro-/pre-T vs IM, cortical-T vs pre-αβ or mature vs TCR-γδ or TCR-β based upon the EGIL[8,31] or TCR classification[23]

criteria (Table 2; Spearman's p=0.474, p<0.001 (two-tailed)). Multiple samples, however, were classified to discordant stages. Twelve IM T-ALLs lacking Cyt-β expression were assigned to the cortical T-cell subgroup based on their CD1 positivity. Eleven pre-αβ T-ALLs were denoted as pro-/preT (seven cases) or mature T-ALLs (four cases) according to EGIL criteria. Seven mature TCR-αβ or TCR-γδ-positive T-ALLs were denoted as cortical-T based upon their CD1 positivity despite the fact that these seven cases expressed mCD3. One TCR-αβ-positive T-ALL was found to be negative for both mCD3 and CD1 and was assigned to the pro-/pre-T subgroup.

Molecular-cytogenetic abnormality distribution over EGIL and TCR subgroups

For all 72 T-ALL patients, the rearrangements status of *TLX3/HOX11L2, TLX1/HOX11, TAL1, CALM-AF10* or *LMO2* genes was determined using FISH and quantitative real-time RT-PCR (RQ-PCR).[12,43] In Table 3, the distribution of these molecular-cytogenetic subgroups over various T-cell developmental stages according to EGIL or TCR classification criteria is shown.

On the basis of EGIL criteria, two out of three *CALM-AF10* rearranged cases were assigned to the pro-/pre-T subgroup. Both patients were also classified as IM T-ALL in accordance to the TCR classification system. One *CALM-AF10* case expressed CD1, and was denoted as cortical-T.

HOX11L2-positive patients predominantly had an IM or cortical-T differentiation stage, and only 4 out of 17 patients had a mature T-cell stage according to EGIL criteria. Using TCR classification criteria, *HOX11L2* rearranged T-ALL cases were classified as IM (7 cases) or pre-αβ (7 cases) and only 3 cases were TCR-γδ positive. None of the *HOX11L2* patients were TCR-αβ positive (p=0.015). All *HOX11*-positive samples expressed CD1 and were therefore assigned to the cortical T-cell subgroup (p=0.008). However, as most *HOX11* patients lacked cytoplasmatic-β (Cyt-β) expression they were assigned to the IM subgroup based upon TCR classification criteria.

The 14 *TAL1*-rearranged cases were equally distributed among EGIL subgroups. However, only four *TAL1*-rearranged cases were classified as pre-αβ based upon Cyt-β expression, but nine *TAL1*-positive cases were TCR positive and exclusively associated with the αβ-lineage (p<0.001). *LMO2* cases were found in all developmental stages of EGIL and TCR classification systems. Both TCR positive, *LMO2*-rearranged cases were TCR-αβ positive comparable to the *TAL1* rearranged cases.

Table 2. Overlap between immunophenotypic classification systems

TCR	EGIL			Total
	Pro-/Pre-T	Cortical	Mature	
IM	14	12	0	26
Pre-AB	7	8	4	19
TCR-GD	0	2	3	5
TCR-AB	1	5	11	17
Undefined	2	3	0	5
Total	24	30	18	72

Abbreviations: IM, immature; n, number.

Table 3. Molecular-cytogenetic profiles in EGIL- and TCR-classifiaction subgroups

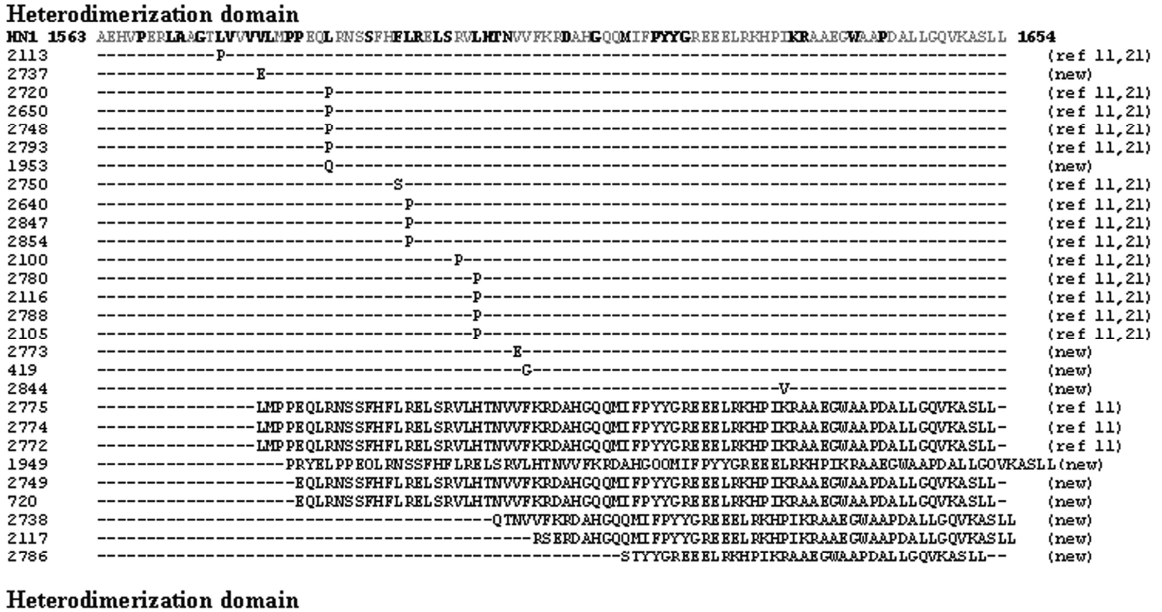
Classification (n)	**Total** (n=72)	***CALM-AF10*** (n=3)	***HOX11L2*** (n=17)	***HOX11*** (n=6)	***TAL1*** (n=14)	***LMO2*** (n=6)	**Other** (n=26)	**Total** (n=70)	***NOTCH1* mutation** (n=40)
EGIL	n	n (%)						*n*	
Pre-T	24	2 (67)	6 (35)	0 (0)	5 (35.5)	1 (17)	10 (38)	24	14(35)
Cortical T	30	1 (33)	7 (41)	6 (100)	5 (35.5)	2 (33)	9 (35)	29	19(48)
Mature T	18	0 (0)	4 (24)	0 (0)	4 (29)	3 (50)	7 (27)	17	7(17)
P-value		NS	NS	p=0.008	NS	NS	NS		NS
TCR (total)								*n*	
Immature	26	2 (67)	7 (41)	4 (67)	0 (0)	1 (17)	12 (46)	26	15(37)
Pre-AB	19	0 (0)	7 (41)	1 (16.5)	4 (29)	2 (33)	5 (19)	19	12(30)
TCRGD	5	0 (0)	3 (18)	0 (0)	0 (0)	0 (0)	2 (8)	4	3(8)
TCRAB	17	0 (0)	0 (0)	0 (0)	9 (64)	2 (33)	6 (23)	16	7(17)
Undefined	5	1(33)	0(0)	1(16.5)	1(7)	1(17)	1 (4)	5	3 (8)
P-value		NS	p=0.015[a]	NS	p<0.001[a]	NS	NS		NS

Abbreviations: EGIL, The European Group for the Immunological Characterization of Leukemias; NS, not significant; TCR, T-cell receptor.
Recurrent molecular-cytogenetic abnormalities including the CALM-AF10 translocation or rearrangements involving the TLX3/HOX11L2, TLX1/HOX11, TAL1 (including SIL-TAL1) or LMO2 (three LMO2 translocated cases as well as three cases with the del(11)(p12p13) leading to the activation of LMO2); other, patients without any of these abnormalities; significant associations as calculated using the Fisher's exact test are shown.
[a] *The undefined groups is left out of the statistical analysis.*

The *NOTCH1* mutation status was successfully determined for 70 out of 72 patients. Mutations in the heterodimerization domain (HD; exon 26 or exon 27) of *NOTCH1* was identified in 28 patients, 6 patients had a PEST (Proline (P), glutamate (E), serine (S) and threonine (T)) domain (exon 34) mutation and another 6 patients had mutations in both the HD and PEST domains (Figures 1a and b). The incidence of *NOTCH1* mutations was less frequent in immunophenotypic mature cases according to both classification systems.

Figure 1

A



B

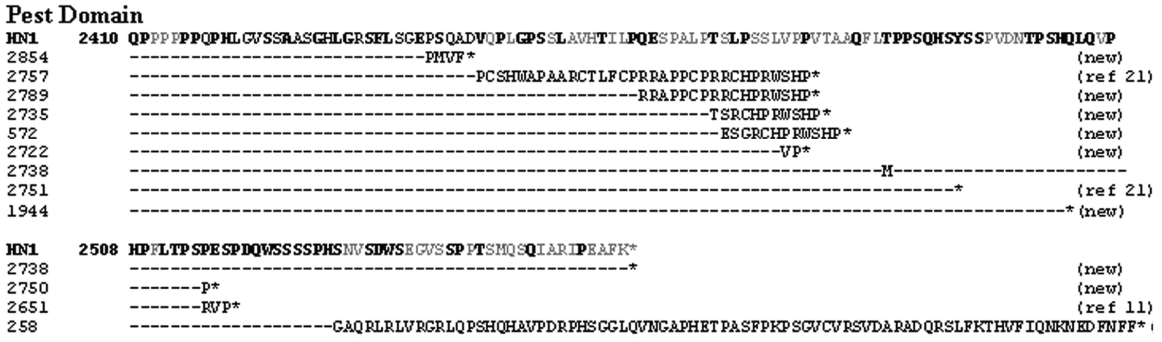


Figure 1. Activating *NOTCH1* mutations in pediatric T-ALL patients
(a) Position of point mutations or in-frame deletion/insertion mutations in the heterodimerization domain (exons 26 and 27) of NOTCH1. Amino-acid numbering is in accordance to Weng et al.[11] Amino-acid residues indicated in bold are conserved amino-acid residues between human, mouse and Xenopus NOTCH 1. (b) The positions of missense and point mutations are given in the PEST domain of NOTCH 1. Asterisks refer to stop codons. T-ALL, T-cell acute lymphoblastic leukemia.

Expression of *TAL1*, *LYL1, LMO1* and *LMO2* in EGIL or TCR-subgroups

In addition to recurrent cytogenetic abnormalities, the expression of several transcription factors including *TAL1*, *LYL1, LMO1* and *LMO2* was investigated in relation to the T-cell developmental subgroups. Rearrangements of the *TAL1*, *LMO1* or *LMO2* loci result in activation of these genes, but various patients also highly express *TAL1*, *LYL1, LMO1* or *LMO2* levels in the absence of chromosomal rearrangements. The expression levels for these transcription factors did not differ between the three EGIL subgroups (data not shown). For the TCR classification subgroups, the levels for *TAL1, LYL1* and *LMO2* were significantly different (Figure 2). The levels of *LYL1* and *LMO2* expression were significantly higher in the IM subgroup than in the pre-αβ (p<0.001 and p=0.005, respectively) and TCR-αβ subgroups (p=0.002 and p=0.003, respectively). *TAL1* expression levels followed an opposite profile with the lowest expression in IM T-ALL cases and the highest levels for the pre-αβ (p=0.002) and TCR-αβ (p=0.003) subgroups.

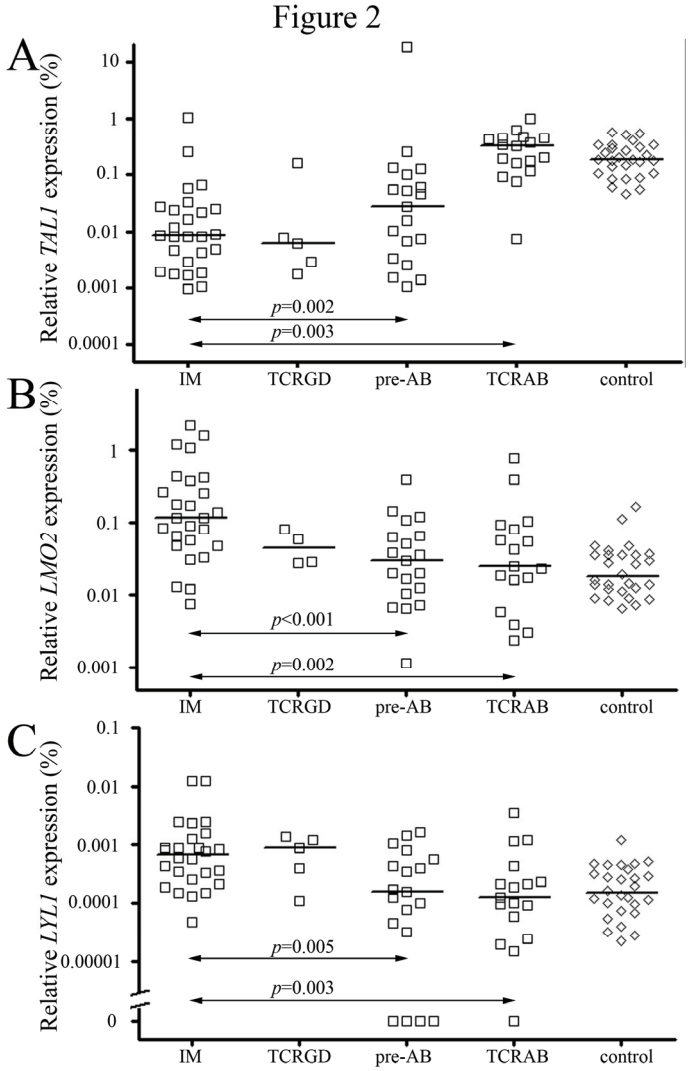


Figure 2. Expression levels of TAL1, LMO2 and LYL1 in TCR classification subgroups

Relative expression levels of (a) TAL1, (b) LMO2 and (c) LYL1 as percentage of housekeeping gene GAPDH expression levels for TCR classification subgroups and bone marrow control samples. All p-values below p=0.008 as indicated between various TCR classification subgroups are significant following the Bonferroni correction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCR, T-cell receptor.

Outcome of T-ALL

Twenty out of 72 patients relapsed (28%). No relation was found between clinical parameters including age, WBC, mediastinal involvement, gender or treatment protocol and DFS (Table 4). None of the EGIL or TCR classification subgroups were associated with outcome (Table 4).

The presence of *HOX11*, *LMO2* abnormalities or *NOTCH1* mutations was not related to DFS (Table 4). *HOX11L2* and *TAL1* abnormalities were associated with a nonsignificant trend toward poor and good DFS, respectively. The three CALM-AF10 rearranged patients had a poor DFS (p=0.0053).

Expression levels of *LYL1, LMO2* and *LMO1* did not predict for outcome. For T-ALL samples expressing comparable *TAL1* levels as *TAL1*-rearranged cases (relative *TAL1* expression levels >0.05%), no difference in outcome was observed compared to cases expressing lower *TAL1* levels. In a trend analysis comparing the outcome of patients with the lowest *TAL1* expression (below the 25th percentile) with patients between the 25th and 50th percentile, between the 50th and 75th percentile and above the 75th percentile of *TAL1* expression, a trend toward better outcome was found for patients with higher *TAL1* expression (p=0.09; Table 4).

DISCUSSION

Molecular-cytogenetic abnormalities as identified in T-ALL may have prognostic significance. *CALM-AF10*-positive T-ALL has been associated with early relapse,[8,12] whereas *HOX11L2* abnormalities have been associated with diverse treatment outcomes depending on the treatment given.[22-26] *HOX11L2* demonstrated a trend to poor outcome in our DCOG pediatric cohort, and was significantly associated with poor outcome for children treated according to the COALL-97 protocol.[12] As molecular-cytogenetic abnormalities are associated with the expression of specific immunophenotypic markers, we have studied whether arrest at specific developmental stages may provide a potential explanation for the observed differences in outcome between various cytogenetic subgroups. To this end, we have classified our T-ALL cases according to the criteria of two currently available T-cell classification systems, that is, the EGIL[26] and the TCR classification system.[23]

Using these classification systems, about half of the T-ALL cases were assigned to more or less comparable developmental stages. However, various samples were assigned to different stages using both classification systems including five TCR-αβ and 2 TCR-γδ-positive cases that were assigned to the cortical T-cell stage using EGIL based on their CD1 expression. All these cases expressed mCD3, and denoting cases as cortical T-cells on the basis of CD1 may be too stringent. The TCR classification system may therefore better relate pediatric T-ALL to normal T-cell development than EGIL. This is also supported by *LYL1, LMO1* and *LMO2* expression levels that were highest for IM T-ALL cases and significant lower levels for pre-αβ and TCR-αβ subgroups alike normal T-cell development.[44,45]

Table 4. Outcome of clinical, immunophenotypic and molecular-cytogenetic subgroups

Parameter	Comparison	5 years DFS (% ± SD)	*p*-value	*p*-value SFP
Clinical (n=72)				
Protocol ALL-7/8 vs ALL-9		69±9 vs 72±7	0.69	
Age	<10 vs >10	62±11 vs 74±6	0.53	0.46
Gender	Male vs Female	66±7 vs 84±8	0.19	0.18
WBC	<50.000 vs >50.000	82±12 vs 68±6	0.32	0.32
Mediastinal involvement	Yes vs No	76±7 vs 66±8	0.31	0.34
Cytogenetic/karyotypic (n=72)				
HOX11 (6/72)	*HOX11+ vs HOX11-*	80±18 vs 70±6	0.54	0.54
HOX11L2 (17/72)	*HOX11L2+ vs HOX11L2-*	57±12 vs 75±6	0.11	0.15
SIL-TAL1/TAL1 (14/72)	*TAL1+ vs TAL1-*	92±8 vs 66±6	**0.075**	**0.076**
LMO2 (6/72)	*LMO2+ vs LMO2-*	50±20 vs 73±6	0.26	0,26
CALM-AF10 (3/72)	*CA+ vs CA-*	0 vs 74±6	**0.0053**	
NOTCH1 mutation (40/70)	mut vs wt	65±8 vs 77±8	0.31	0.32
Transcription factors (relative expression levels)				
TAL1 (n=72)	>0.05 vs <0.05%[a]	72±8 vs 70±7	0.88	0.92
TAL1 (n=72)	<25 vs 25-50 vs 50-75 vs >75%	82±9 vs 46±12	**0.09**	
LYL1 (n=72)	>median vs <median	63±8 vs 80±7	0.13	0.13
LYL1 (n=72)	<25 vs 25-50 vs 50-75 vs >75%	65±11 vs 82±10	0.19	
LMO2 (n=71)	>0.1 vs <0.1%[b]	75±9 vs 69±7	0.48	0.53
LMO2 (n=71)	<25 vs 25-50 vs 50-75 vs >75%	67±11 vs 56±13	0,34	
LMO1 (n=71)	pos vs neg	68±8 vs 74±8	0.64	0.66
EGIL-classification (n=72)				
Pre-/Pro-T	Pre/Pro-T vs other	79±8 vs 66±7	0.32	0.33
Cortical-T	Cortical-T vs other	68±9 vs 73±7	0.71	0.66
Mature	Mature vs other	65±12 vs 73±6	0,49	0.55
TCR-classification (n=67)				
IM	IM vs other	73±9 vs 69±7	0.70	0,74
Pre-αβ	Pre-αβ vs other	73±10 vs 69±7	0,81	0,86
TCRαβ	TCRαβ+ vs other	69±12 vs 72±6	0.77	0,83
TCRγδ	TCRγδ+ vs other	60±22 vs 72±6	0.48	0.56

Abbreviations: DFS, disease-free survival; F, female; M, male; SFP, stratified for protocol.
[a] Expression level equivalent to the lowest TAL1 level for TAL1-rearranged patients.
[b] Expression level equivalent to the lowest LMO2 level for LMO2-rearranged patients.
Significant and nearly significant p-values have been indicated in bold.

All *HOX11*-rearranged cases in our study expressed CD1 and were denoted as cortical thymocytes using EGIL (p=0.008). As four out of five cases lacked Cyt-β expression, developmental arrest for *HOX11* T-ALL patients precedes β-selection in most patients. *HOX11L2*-positive cases were predominantly associated with the pre-T/IM and cortical-T/pre-αβ subgroups. A few *HOX11L2* cases had a mature phenotype but exclusively associated with the TCR-γδ lineage. On the other hand, expression of Cyt-β in seven cases may indicate partial commitment to the αβ-lineage in support of the hypothesis by Asnafi et al.[23] that *HOX11L2*-rearranged T-ALL cases resemble αβ-lineage cortical thymocytes that are differentiated toward unusual TCR-γδ expressing cells as intermediates between the αβ-lineage and the γδ-lineage. *TAL1*-rearranged cases were associated with all maturation stages according to EGIL, but were not

associated with IM subtype nor with γδ-lineage commitment according to the TCR classification system in accordance with previous studies.[8,24,25] *LMO2*-rearranged cases including the three cases with the newly identified del(11)(p12p13) leading to elevated *LMO2* levels[43] seem to arrest at all stages of T-cell maturation stages based on EGIL and the TCR classification criteria except for the γδ-lineage. As *TAL1* and *LMO2* participate in the same transcriptional complex,[46-48] we would have expected a similar distribution for *TAL1* and *LMO2* cases. On the other hand, observed differences may be due to the low numbers of *LMO2*-rearranged patient in our cohort.

Activating *NOTCH1* mutations were present in 57% of T-ALL patients in line with previous reports.[11,21] Eleven out of 12 PEST domain mutations were missense mutations leading to truncation of NOTCH1 and deletion or disruption of the negative regulatory PEST domain.[11] One mutation was a point mutation of a conserved amino-acid residue in NOTCH1, and we speculate that this point mutation will interfere with the function of the PEST domain. The 34 mutations in the heterodimerization domain were all point mutations or in-frame deletion/insertion mutations, possibly promoting ligand-independent protease cleavage and release of intracellular NOTCH1 (ICN).[11] Nine out of 12 PEST domain mutations and 14 out of 34 HD mutations were new and have not been observed before in other T-ALL patient cohorts.[11,21] *NOTCH1* mutations were predominantly associated with the cortical stage in another study.[21] We did not observe significant differences in the distribution of *NOTCH1* mutations between EGIL or TCR classification subgroups, in line with the presence of *NOTCH1* mutations in all molecular-cytogenetic T-ALL subgroups. We also did not observe any differences regarding outcome in contrast to that same study in which activating *NOTCH1* mutations were associated with favorable treatment response and long-term outcome in pediatric T-ALL.[21]

None of the EGIL or TCR classification subgroups predicted for outcome. Pro-/pre-T or IM immunophenotypes have been previously associated with reduced-remission induction[2,27,30,31] and poor outcome[2,29] in adult T-ALL. These observations could not be confirmed in our pediatric T-ALL cohort. Our *HOX11*-rearranged patients that were all CD1 positive did not have an improved outcome as published,[14,18-20,28,32] but the number of *HOX11*-rearranged patients in our study is low. As described earlier[12] and now analyzed over a prolonged follow-up, *HOX11L2* demonstrated a trend towards poor outcome in our pediatric cohort.[12] Within this subgroup, CD1-positive cases relapsed as frequently as CD1-negative cases.[12]

TAL1 rearranged T-ALL demonstrated a trend for good outcome, in line with one study[14] but in contrast to another study.[34] This was supported by analyzing the outcome of patients based on *TAL1* expression levels in which cases with higher *TAL1* levels demonstrated a trend for good outcome. Alternatively, the 18 cases with the lowest *TAL1* levels included 10 *HOX11L2*-rearranged and 2 *CALM-AF10*-positive T-ALL cases, two cytogenetic entities that both have been associated with poor outcome in other studies.[8,13,34]

In conclusion, the present study shows that differences in outcome for various molecular-cytogenetic subgroups cannot be attributed to differences in T-cell maturation stage.

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5

CD34 expression is associated with poor survival in pediatric T-cell acute lymphoblastic leukemia

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ABSTRACT

Background
Children with T-lineage acute lymphoblastic leukemia (T-ALL) have an inferior outcome with combination chemotherapy compared to B-lineage ALL, and still about 30 percent of the patients relapse within the first 2 years following diagnosis. As CD34 has been related with poor outcome in ALL in general, we investigated the prognostic significance of the stem cell marker CD34, as well as the association of CD34 positivity with the expression of several multidrug resistance (MDR) genes.

Procedure
In this retrospective study, we investigated the prognostic significance of the expression of the early T-cell differentiation marker CD34 and the expression of MDR genes in relation to outcome in a cohort of 72 newly diagnosed pediatric T-ALL patients.

Results
CD34 expression was related to a poor 5 year disease-free-survival and a poor 5 year overall survival. Using the Cox proportional hazard model, CD34 expression predicted for increased risk for relapse and death. Expression of CD34 was associated with elevated *MDR1* and *MRP1* mRNA expression levels. For the entire T-ALL cohort, these expression levels of *MDR1* or *MRP1* did not independently predict for poor outcome.

Conclusions
We conclude that CD34-positive T-ALL has a relatively poor survival that is not explained by the mRNA expression levels of *MDR1*, *LRP* or *MRP1*.

INTRODUCTION

The outcome of pediatric acute lymphoblastic leukemia (ALL) has improved over the last decades. The 5 year survival for precursor B-lineage ALL reaches 80 percent using current treatment protocols. T-lineage ALL still has a relatively poor outcome with a 5 year survival of 60-70 percent.[1]

For acute leukemia in general, expression of the stem-cell marker CD34 has been associated with elevated levels of multidrug resistance (MDR) genes and to a poor response to chemotherapy.[2-4] It was concluded that expression of MDR genes in these ALL subsets may provide a plausible explanation for poor outcome. However, as most of these studies were based on cohorts comprising both adult and pediatric patients of predominantly the B-lineage subtype, still little is known about the prognostic significance of the expression of multidrug resistance genes for pediatric T-ALL patients.

Pui *et al*. did not observe a significant adverse prognostic effect for CD34 expression in 61 pediatric T-ALL patients.[5] Also, conflicting data have been reported about the prognostic role of various MDR genes in childhood ALL, like *MDR1/ABCB1/GP170*, multidrug resistance-associated protein 1 (*MRP1/ABCC1*), the lung resistance protein (*LRP*; or major vault protein *MVP*) and the breast cancer resistance protein (*BCRP/ABCG2*).[6] Several studies show an association between high P-glycoprotein (P-gp) expression, encoded by the *MDR1* gene, with poor clinical outcome,[7,8] whereas other studies contradict these findings.[9-11] In a study, comprising 295 pediatric ALL patients, *MRP1* overexpression was associated with T-cell immunophenotype whereas an increased *LRP* expression was found in B-lineage ALL. Overexpression of these drug efflux pumps at diagnosis did not predict for treatment failure.[12]

The relevance of early differentiation markers as well as the role of multiple drug resistance genes in relation to outcome for pediatric T-ALL remains unclear. We therefore investigated the prognostic significance of the expression of early differentiation marker CD34 and the expression of MDR genes in pediatric T-ALL.

METHODS

Patients

Viably frozen diagnostic bone marrow (n=41) or peripheral blood samples (n=31) were obtained from 72 pediatric T-ALL patients, diagnosed between 1991 and 2000 and treated according to the national protocols DCOG ALL-7 (n=4), ALL-8 (n=26) or ALL-9 (n=42). The median follow-up time was 63.5 months. Patient samples were processed as described previously.[13] Upon thawing of viably frozen patient samples, all patient samples were enriched to contain >90% of leukemic blasts, as described.[13] Informed consent from parents and patients was obtained according to the declaration of Helsinki.

CD34 expression

The central reference laboratory of the DCOG performed the immunophenotyping of fresh diagnostic patient material for the various markers mentioned following a one-step standard procedure as described earlier, including CD34.[14] CD7, CD3 monoclonal antibodies were purchased at Becton Dickinson (San Jose, CA, USA) in conjugation to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) fluorochromes. All T-ALL patients were CD7 and cytoplasmic CD3 positive. In this study, patients were classified as positive for each immunophenotypic marker indicated when ≥25% of the leukemic blasts expressed this specific immunophenotypic marker, or indicated as negative when expressed in <25% of the leukemic blasts. As positivity levels for immunophenotypic markers are frequently determined at the 20% positivity level, we also tested the significance of CD34 expression at the 20% cut-off as clearly specified in the text.

Quantitative real-time RT-PCR (RQ-PCR) for *MDR1*, *MRP1* and *LRP* mRNA expression levels

mRNA extraction and reverse transcription were performed as described previously.[13] The mRNA expression levels of the drug resistance genes *MDR1*, *MRP1* and *LRP* were quantified relative to the expression level of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by real-time RT-PCR in an ABI 7700 sequence detection system (PE Applied Biosystems, Foster-city CA, USA).[13,15,16] Positivity and negativity for expression of these genes was defined at three different cut-off levels: above or below the median, above or below the 25 percentile of expression, or above or below the 75 percentile of expression.

Statistical analysis

Kaplan Meier curves were constructed in SPSS 11.0 software, and *p*-values for DFS and overall survival (OS), were determined using the log-rank test ($p_{log\text{-}rank}$). For calculation of DFS, non-response to induction therapy or relapse was defined as an event. The endpoint for the survival analyses was death from any cause. The Fisher exact test was used to test a difference in the distribution of CD-marker over different cytogenetic subgroups (p_{Fisher}). The Mann-Whitney U-test (MWU) was used to test the relation between the CD marker and relative *MDR1, MRP1* and *LRP* expression levels (p_{MWU}). The Cox proportional hazard model was used to calculate potential associations with increased risk for relapse or death in univariate or multivariate analyses (p_{Cox})

RESULTS AND DISCUSSION

The clinical characteristics of the patients have been reported previously,[17] and are summarized in the Supplementary Table 1. Twenty out of 72 patients relapsed. Thirty patients were treated with the equivalent protocols ALL-7 and ALL-8, whereas 42 patients were treated according to the ALL-9 protocol. Since the outcome for the ALL 7/8 versus ALL-9 treated patients was not different (Table 1), these DCOG patients were regarded as a single cohort in this study unless indicated. No relationship was

observed for age, WBC or gender with the 5 year disease free survival (DFS) or 5 year survival (Table 1). Also, no significant results were obtained when stratified for treatment protocol. In our previous study, these factors were not associated with an increased risk for relapse or death using the Cox regression analysis.[17]

When analyzing the prognostic impact of T-cell development stage for pediatric T-ALL in our previous study, we did not observe an association for any differentiation stage according to EGIL or TCR classification criteria and outcome.[18] When further extended this analysis by investigating the prognostic impact of various individual CD markers including the stem-cell marker CD34, the myeloid markers CD33 and/or CD13, CD1 or CD10. We observed that expression of CD34, as identified in 18 patients, was significantly associated with a poor 5 year DFS ($CD34^+$ versus $CD34^-$: 52±12% versus 77±6%; $p_{log\text{-}rank=}0.05$; Table 1) and a poor overall survival (OS) ($CD34^+$ versus $CD34^-$: 39±11% versus 78±6%; $p_{log\text{-}rank=}0.0013$; Table 1 and Figure 1), even following stratification for treatment protocol (SFP; 5 year EFS is $p_{log\text{-}rank=}0.04$). As we used a cut-off at the 25% positivity level throughout this study for the expression of immunophenotypic markers, CD34 was also associated with poor outcome at the cut-off level of 20% such as has been used in other studies (5 year DFS: $CD34^+$ (n=23) versus $CD34^-$ (n=49): 50±11% versus 81±6%; $p_{log\text{-}rank=}0.0042$). When calculating the risk for CD34 for increased relapse and death in a univariate analysis using the Cox proportional hazard model at either positivity level, CD34 predicted a 2-3 fold higher risk for relapse, and for a 3.5-4.5 fold higher risk for death (Table 2).

Table 1. Disease free survival in pediatric T-ALL

Parameter		**5 years DFS (% ± SD%)**	$p_{log\text{-}rank}$ **DFS**	$p_{log\text{-}rank}$ **DFS SFP**
Clinical				
Age	<10 vs >10	62±11 vs 74±6	0.53	0.46
WBC	<50 vs >50	82±12 vs 68± 6	0.32	0.32
Gender	male vs female	66±7 vs 84±8	0.19	0.18
Mediastinal involvement	yes vs no	76±7 vs 66±8	0.31	0.34
Protocol	ALL7/8 vs ALL9	69±9 vs 72±7	0.69	
Molecular-cytogenetic				
NOTCH1	mutant vs wildtype	62±8 vs 82±7	0.08	0.1
Immunophenotype				
CD34	CD34+ vs CD34-	52±12 vs 77±6	**0.05**	**0.04**
CD33/CD13	CD33/13+ vs CD33/13-	56±15 vs 74±6	0.2	0.14
CD1	CD1+ vs CD1-	68±9 vs 73±7	0.71	0.66
CD10	CD10 + vs CD10-	65±10 vs 74±6	0.49	0.54
Multidrug resistance genes (relative expression)				
MDR1	<median vs >median	67±8 vs 73±8	0.64	0.68
MRP1	<median vs >median	76±7 vs 64±8	0.29	0.32
LRP	<median vs >median	79±7 vs 62±8	0.14	0.14

SD, standard deviation; Age, indicated in years; WBC, white blood cell count ($x10^9$ leukoctes/L); vs, versus; DFS, disease free survival; SFP, stratified for protocol.

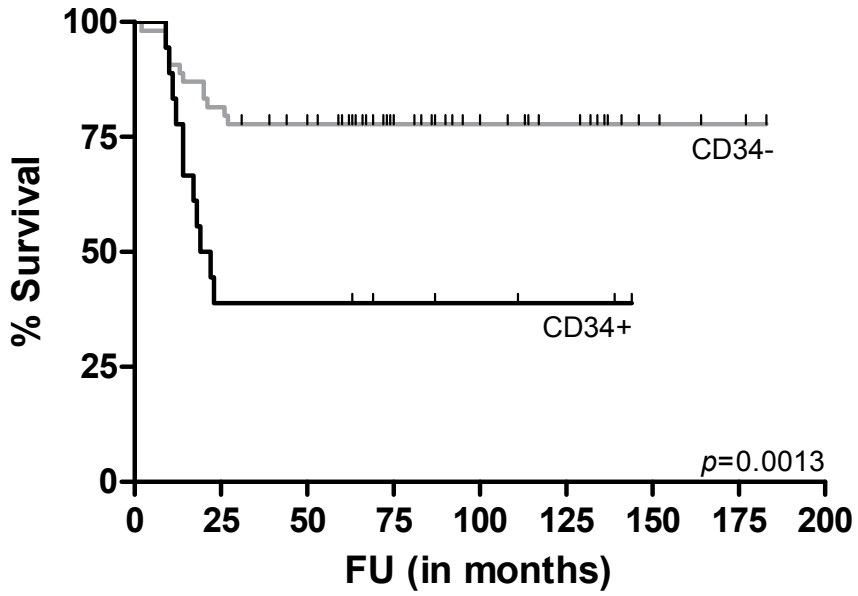

Figure 1. Relation between CD34 and survival in pediatric T-ALL
The p-value for the Kaplan-Meier curves is calculated using the log-rank test. The 5 year OS for CD34$^+$ T-ALL (n=18) versus CD34$^-$ T-ALL (n=54) is 39±11% versus 78±6%.

CD34 is a marker that is normally expressed on hematopoietic stemcells, and early thymic T-cell precursors, therefore, expression of CD34 could reflect early T-cell maturational arrest in T-ALL.[5,19] However, expression of CD34 is not associated with an immature immunophenotype according to TCR classification criteria ($p_{Fisher=}$0.823). CD34 was associated with immature as well as mature development stages but was less frequently associated with the intermediate cortical T-cell stage based upon EGIL criteria ($p_{Fisher=}$0.005).

CD34 was also not associated with the presence of specific molecular-cytogenetic abnormalities as present in our cohort (n=72) that might have explained the association with poor outcome, including *TAL1/SCL* (n=14), *HOX11/TLX1* (n=6), *HOX11L2/TLX3* (n=17/), *CALM-AF10* (n=3) or the absence of these abnormalities (n=32) ($p_{Fisher=}$0.22). Nor was expression of CD34 associated with the presence or absence of activating *NOTCH1* mutations ($p_{Fisher=}$0.57). Activating *NOTCH1* mutations[20] including the recently identified insertions in exon 28 [21] as identified in patients # 1946 and # 1950 were not associated with outcome in this cohort (Table 1).

Expression of CD34 has previously been associated with elevated *MDR1* mRNA expression in adult T-ALL.[2-4] We therefore investigated whether expression of the multiple drug resistance genes *MDR1*, *MRP1* or *LRP* could provide an explanation for this relationship between CD34 and outcome. Expression of CD34 appeared to be significantly associated with elevated mRNA levels of *MDR1* ($p_{MWU=}$0.01) and *MRP1* ($p_{MWU=}$0.019), but not *LRP* ($p_{MWU=}$0.31).

We then investigated whether mRNA expression of these *MDR* genes was related to outcome. Whatever cut-off level for these expression levels used, *MDR1*, *MRP1* and *LRP* mRNA expression levels were not related to outcome (Table 1). As the high-risk treatment arm for the ALL7/8 and ALL-9 protocols that were used for T-ALL patients contain comparable cumulative doses of anthracyclines, this may explain why no significant differences in outcome were identified following stratification for treatment

protocol. When analysing CD34-positive patients only (n=18), the outcome of the patients with *MDR1* expression above the median (n=9) was not different from patients with *MDR1* expression below the median (n=9; DFS, $p_{log\text{-}rank=}$0.91, OS, $p_{log\text{-}rank=}$0.73). In a univariate analysis, *MDR1* nor *MRP1* mRNA expression levels were associated with an increased risk for relapse and death (Table 2). In multivariate analysis, the increased risk for relapse and death for the expression of CD34 was independent from MDR1 and MRP1 expression (Table 2).

This study shows that high mRNA expression levels of multidrug resistance genes may not explain the observed association between CD34 expression and poor outcome in this cohort of T-ALL patients. It could be that *MDR* expression levels may not directly relate to MDR protein function, and MDR1 function has been associated with reduced complete remission induction in adult T-ALL patients treated on the GIMEMA LAL0496 protocol.[3] As MDR function needs to be established on fresh patient material, we could not study MDR function in relation to outcome as part of this study. In conclusion, pediatric T-ALL cases expressing the early differentiation marker CD34 are associated with poor DFS and survival. CD34 was also associated with higher mRNA expression levels of *MDR1* and *MRP1*. The expression levels of these multiple drug resistance genes are not associated with outcome, and therefore do not explain the poor prognostic impact of CD34 expression.

Table 2. CD34 expression predicts for increased risk for relapse and death

Univariate analysis for DFS marker		**n**	**HR**	**CI**	***p cox***
CD34	>25%	18	2.339	0.953-5.740	**0.064**
CD34	>20%	23	3.352	1.384-8.118	**0.007**
MDR1	above the median	36	0.814	0.337-1.965	0.647
MRP1	above the median	36	1.602	0.655-3.921	0.302
Univariate analysis for OS marker					
CD34	>25%	18	3.538	1.551-8.072	**0.003**
CD34	>20%	23	4.516	1.941-10.505	**<0.001**
MDR1	above the median	36	0.932	0.411-2.112	0.866
MRP1	above the median	36	1.438	0.630-3.282	0.388
Multivariate analysis for DFS marker					
CD34	>20%	18	3.301	1.263-8.624	**0.015**
MDR1	above the median	36	0.466	0.170-1.275	0.137
MRP1	above the median	36	1.591	0.456-4.555	0.387

Univariate and multivariate analyses using the Cox proportional hazard model for CD34 expression at the 25% or 20% positivity levels, and MDR1 and MRP1 expression (above the median). n, number of patients; HR, hazard ratio; CI, 95% confidence interval; DFS, disease free survival; OS, overall survival.

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Supplementary Table 1. Summary of clinical, immunophenotypic and molecular cytogenetic parameters for pediatric T-ALL patients treated on DCOG protocols

Patient nr	M/F	Age (yrs)	WBC x10e9/l	Protocol	CD7	CD2	CD5	CD1	CD10	CD34	CD33	CD13	cytβ	CD4	CD8	smCD3	TCR αβ	TCR γδ	EGIL class.	TCR class.	T-ALL subgroup	*NOTCH1* status	Karyotype	Relapse CCR	FU DFS	Status
1949	M	14,9	300	ALL8	99	99	98	13	6	74	0	0	ND	11	84	73	αβ	neg	Mature	TCRαβ	TAL1	mutated	46,XY[19]	CCR	139	alive
2722	M	11,3	192	ALL8	91	91	90	12	0	36	0	0	pos	81	64	7	neg	neg	Pro-/pre	Pre-αβ	TAL1	mutated	46,XY,t(1;7)(p31;q32)(52%)/46,XY (48%)	CCR	111	alive
720	M	3,2	417	ALL9	97	73	96	11	0	39	0	0	pos	28	0	1	neg	neg	Pro-/pre	Pre-αβ	HOX11L2	mutated	46,XY,t(7;9)(p1?3;p2?2)[30]/46,XY[4]	CCR	63	alive
1946	M	4,5	405	ALL8	97	33	98	4	94	27	0	1	ND	80	76	91	neg	γδ	Mature	TCRγδ	HOX11L2	mutated	46,XY	R	12	dod
2640	M	6,2	68,9	ALL8	98	98	98	0	94	37	0	0	pos	86	0	6	neg	neg	Pro-/pre	Pre-αβ	HOX11L2	mutated	46,XY[10]	R	11	dod
2723	F	6,4	98	ALL8	98	28	98	0	0	30	24	47	ND	75	46	9	neg	neg	Pro-/pre	IM	HOX11L2	mutated	45,XX,dic(7;8)(p12;p12)[7]	R	8	dod
2738	F	12,3	34,1	ALL9	95	68	93	2	90	81	34	26	neg	0	1	27	neg	neg	Mature	Pre-αβ	HOX11L2	mutated	46,XX,del(9)(p2?1)[9]/46,XX,add(9)(p12)[6]	CCR	11	td
2651	M	10,8	347	ALL9	99	99	99	38	0	92	78	0	ND	0	0	1	neg	neg	Cortical	ND	CALM-AF10	mutated	46,XY,t(10;11)(p13;q21)[20]	R	22	dod
2736	M	10,1	13,7	ALL9	96	0	2	0	44	69	44	2	neg	1	1	2	neg	neg	Pro-/pre	IM	CALM-AF10	wt	46,XY,t(2;9)(q21;q34),?t(8;8)(?q22;q?24)[18]/46,XY[6]	R	19	dod
2649	F	16,7	86,3	ALL9	81	83	79	12	0	61	15	13	neg	21	58	81	αβ	neg	Mature	TCRαβ	UNKN	wt	46,XX,del(6)(q13q21),t(8;14)(q24;q11),del(9)(p22)	CCR	22	td
2779	F	2,8	57,6	ALL8	98	98	97	16	13	48	1	0	neg	22	63	43	αβ	neg	Mature	TCRαβ	UNKN	wt	46,XX,del(6)(q?)[8]	CCR	144	alive
335	M	13,9	600	ALL9	95	98	95	74	1	90	0	0	pos	52	43	95	neg	neg	Cortical	Pre-αβ	UNKN	wt	46,XY[26]	R	14	dod
572	F	8,4	15,4	ALL9	97	15	96	0	4	59	0	0	neg	3	2	5	neg	neg	Pro-/pre	IM	UNKN	mutated	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]	CCR	63	alive
2703	M	3,9	435	ALL9	94	94	92	8	0	79	0	3	neg	0	1	2	neg	neg	Pro-/pre	IM	UNKN	wt	46,XY[30]	CCR	18	td
2845	M	1,8	250	ALL9	73	1	66	0	0	30	0	10	pos	2	2	56	neg	neg	Mature	Pre-αβ	UNKN	wt	46,XY,t(X;11)(q2?4;p13),add(3)(p24)[26]	CCR	69	alive
2852	M	13,8	128,9	ALL9	82	81	82	0	0	66	0	0	pos	21	14	80	αβ	neg	Mature	TCRαβ	UNKN	wt	46,XY,t(8;14)(q24;q11)[14]	R	13	dod
2748	M	1,3	177	ALL9	95	3	92	0	50	72	2	0	neg	2	1	89	neg	γδ	Mature	TCRγδ	UNKN	mutated	46,XY[31]	R	8	dod
2793	F	5,3	60	ALL9	95	4	91	0	2	89	81	69	neg	0	1	17	neg	neg	Pro-/pre	IM	UNKN	mutated	47~48,XX,add(1)(p36)[4],+add(1)(p36)[3],-5[2],del(6)(q?)[2],-18[2],+mar[2][cp4]	CCR	87	alive
258	M	7,5	130	ALL9	92	91	90	0	0	0	6	6	pos	66	63	14	neg	neg	Pro-/pre	Pre-αβ	TAL1	mutated	46,XY[32]	CCR	75	alive
531	F	8	132	ALL9	77	78	77	0	0	0	17	16	ND	11	31	8	neg	neg	Pro-/pre	ND	TAL1	wt	46,XX,?add(9)(p1?)[6]/46,XX[30]	CCR	64	alive
1941	M	11,9	110	ALL7	93	96	87	0	0	0	0	ND	ND	5	8	63	αβ	neg	Mature	TCRαβ	TAL1	wt	46,XY,del(6)(q1?q2?),del(9)(p?)[19]/ 46,XY,t(1,19)(q23,p13),del(9)(p1?)[3]	CCR	183	alive
1960	M	4,3	205	ALL8	99	98	87	10	0	21	0	21	ND	9	31	i	αβ	neg	Mature	TCRαβ	TAL1	ND	45,XY,del(6)(q15q25),dic(9;13)(p11;q13)[34]	CCR	141	alive
2720	M	15,4	200	ALL8	96	97	96	7	80	0	0	0	pos	83	87	90	αβ	neg	Mature	TCRαβ	TAL1	mutated	46,XY,t(1;14)(p32;q11)/47,XY,t(1;14)(p32;q11),+mar	R	14	alive
2751	F	9,8	27,6	ALL9	96	ND	93	85	10	1	3	3	pos	60	92	60	neg	neg	Cortical	Pre-αβ	TAL1	mutated	45,XX,der(9)t(9;14)(p13;q1?2),del(12)(p12p13),-14,idic(17)(p11)[38]	CCR	86	alive
2759	M	3,3	590	ALL9	93	94	91	0	1	16	4	3	pos	3	1	4	neg	neg	Pro-/pre	Pre-αβ	TAL1	wt	46,XY[30]	CCR	72	alive
2760	M	10,4	112,2	ALL9	92	90	90	59	0	0	0	0	neg	74	70	49	αβ	neg	Cortical	TCRαβ	TAL1	wt	46,XY,dup(17)(q12q24)[14]	CCR	67	alive
2774	M	13,4	41,4	ALL9	61	64	64	0	49	0	0	25	pos	63	64	15	αβ	neg	Pro-/pre	TCRαβ	TAL1	mutated	45,XY,-14[13]/46,XY[16]	CCR	31	alive
2788	M	9,3	310	ALL9	96	90	93	27	0	16	0	0	pos	77	77	87	αβ	neg	Cortical	TCRαβ	TAL1	mutated	46,XY,t(1;14)(p32;q11)inc[2]	CCR	114	alive
2844	F	13	95	ALL9	68	72	68	27	3	0	20	25	pos	14	10	76	αβ	neg	Cortical	TCRαβ	TAL1	mutated	46,XX[28]	CCR	2	td
2854	M	6,4	188,1	ALL9	93	92	92	51	1	0	2	3	pos	24	21	91	αβ	neg	Cortical	TCRαβ	TAL1	mutated	46,XY[30]	CCR	92	alive
634	M	5,5	185	ALL9	89	86	88	43	37	15	8	7	neg	79	49	1	neg	neg	Cortical	IM	HOX11L2	wt	46,XY,ins(5)(q3p1?4p1?2)[15]/46,XY[4]	CCR	62	alive
1179	M	5,9	276	ALL9	99	88	98	27	1	21	21	0	neg	60	0	0	neg	neg	Cortical	IM	HOX11L2	wt	46,XY,del(11)(q2?1q2?4)[21]/46,XY,add(9)(q11),del(11)(q2?1q2?4)[4]	R	13	dod
1954	F	7,8	89,5	ALL8	91	90	90	90	90	2	9	2	ND	47	78	89	neg	γδ	Cortical	TCRγδ	HOX11L2	ND	46,XX[20]	CCR	136	alive
2100	M	5,8	500	ALL7	44	78	84	1	6	0	0	ND	ND	38	4	96	neg	γδ	Mature	TCRγδ	HOX11L2	mutated	UNKN	CCR	50	alive
2105	M	5	140	ALL8	96	96	96	87	92	0	0	0	ND	86	91	4	neg	neg	Cortical	Pre-αβ	HOX11L2	mutated	46,XY(100%)	R	12	dod
2112	M	7,2	354	ALL8	98	72	98	0	8	24	0	24	ND	71	23	1	neg	neg	Pro-/pre	IM	HOX11L2	wt	46,XY[6]	CCR	100	alive
2650	M	6,3	35,9	ALL9	97	4	93	47	0	0	0	0	neg	69	68	0	neg	neg	Cortical	IM	HOX11L2	mutated	46,XY,add(5)(q3?5),-14,+mar[17]	CCR	87	alive
2757	M	6,3	174	ALL9	94	90	70	91	2	11	72	2	pos	71	24	2	neg	neg	Cortical	Pre-αβ	HOX11L2	mutated	46,XY,t(1;7)(p34;q34)[15]	R	9	dod
2772	M	5,1	80	ALL9	90	83	90	0	0	0	0	0	neg	65	1	2	neg	neg	Pro-/pre	IM	HOX11L2	mutated	46,XY[30]	CCR	83	alive
2773	M	5,3	31,8	ALL9	85	86	83	77	0	0	0	0	pos	81	1	18	neg	neg	Cortical	Pre-αβ	HOX11L2	mutated	46,XY[32]	CCR	81	alive
2780	F	8,8	88,5	ALL8	66	13	70	6	59	7	28	28	neg	57	7	48	neg	neg	Mature	Pre-αβ	HOX11L2	mutated	46,XX(100%)	CCR	132	alive
2786	M	5,1	44,9	ALL8	83	85	83	0	0	1	0	11	neg	80	3	3	neg	neg	Pro-/pre	IM	HOX11L2	mutated	47,XY,+8[4]	R	17	dod

419	M	10,3	149	ALL9	81	67	86	59	78	0	0	8	neg	70	69	10	neg	neg	Cortical	IM	HOX11	mutated	46,XY,t(10;14)(q2?4;q11)[2]/46,idem,del(12)(p11)[10]/46,idem, t(6;7)(p21;q34~35),del(12)(p11)[3]/46,XY[9]	CCR	74	alive
1944	F	4,4	280	ALL7	96	92	98	25	0	0	0	ND	ND	91	12	3	neg	neg	Cortical	ND	HOX11	mutated	46,XX	CCR	9	td
1955	M	7,8	159	ALL8	96	68	95	45	88	0	0	0	ND	85	85	4	neg	neg	Cortical	IM	HOX11	mutated	46,XY,t(7;10)(q36;q25-q26)	CCR	137	alive
2737	M	11,2	53,9	ALL9	97	98	97	98	96	0	0	0	neg	95	68	20	neg	neg	Cortical	IM	HOX11	mutated	46,XY,t(10;14)(q24;q11)[24]	R	37	alive
2781	M	9,9	27,2	ALL8	88	89	88	86	10	0	0	0	neg	80	36	0	neg	neg	Cortical	IM	HOX11	wt	46,XY[32]	CCR	95	alive
2792	M	3,5	56,6	ALL9	92	92	89	83	9	1	2	7	neg	78	3	34	neg	neg	Cortical	Pre-αβ	HOX11	wt	47,XY,+del(9)(p1?1)[11]	CCR	74	alive
2750	M	7,9	124,1	ALL9	95	3	94	0	15	0	28	0	neg	69	1	0	neg	neg	Pro-/pre	IM	CALM-AF10	mutated	46,XY,del(9)(p13p22),t(10;11)(p13;q21)[30]	R	9	dod
540	F	2,2	153	ALL9	93	91	91	17	0	0	2	5	pos	81	40	12	neg	neg	Pro-/pre	Pre-αβ	MLL	wt	46,XX,t(6;11)(q27;q23)[10]/47,XX,t(6;11)(q27;q23),+8[6]/46,XX[8]	CCR	69	alive
2847	F	12	41,3	ALL9	85	3	85	0	83	0	9	10	neg	35	1	2	neg	neg	Pro-/pre	IM	MLL	mutated	48,XX,t(6;11)(q27;q23),+i(9)(q10),del(12)(p11),+21	CCR	60	alive
1950	F	9,3	900	ALL8	99	99	98	0	13	0	0	0	ND	85	91	80	αβ	neg	Mature	TCRαβ	LMO2	mutated	46,XX,del(9)(p13p23)/46,XX,del(9), del(13)(q14q22)/46,XX,del(6)(q13q23),del(9)/46,XX,del(6),del(9),del(13)	R	16	dod
2104	M	2,8	649	ALL7	94	98	99	1	0	0	0	ND	ND	6	1	45	αβ	neg	Mature	TCRαβ	LMO2	wt	UNKN	CCR	177	alive
2698	M	6,2	191	ALL9	90	90	90	0	1	1	8	5	ND	42	69	3	neg	neg	Pro-/pre	ND	LMO2	wt	46,XY[30]	CCR	73	alive
2735	F	4,7	212	ALL8	97	97	96	71	32	4	1	1	neg	90	94	8	neg	neg	Cortical	IM	LMO2	mutated	46,XX[32]	R	9	dod
2789	M	9	95,2	ALL9	82	80	84	25	0	1	12	5	pos	59	73	12	neg	neg	Cortical	Pre-αβ	LMO2	mutated	46,XY,t(11;14)(p13;q11)[10]	R	18	dod
2846	M	2,3	124,5	ALL9	73	73	74	0	23	0	10	7	pos	48	57	26	neg	neg	Mature	Pre-αβ	LMO2	wt	46,XY[20]	CCR	39	alive
914	M	1,5	135	ALL9	94	94	85	84	0	0	4	0	neg	50	12	3	neg	neg	Cortical	IM	UNKN	wt	46,XY,del(6)(q1?4q2?2),t(6;7)(q24;q35)[16]/46,XY	CCR	63	alive
2702	M	15,7	128,7	ALL9	95	57	92	77	92	0	0	35	neg	2	78	2	neg	neg	Cortical	IM	UNKN	mutated	46,XY,del(6)(q15q21),del(14)(q11q32), der(14)inv(14)/46,idem,dup(1)?(q21q42)[2]/46,idem,del(17)(p11)[4]	CCR	53	alive
2775	M	15,9	136	ALL9	83	85	80	0	0	22	11	0	pos	5	2	74	αβ	neg	Mature	TCRαβ	UNKN	mutated	46,XY,del(6)(q2?q2?)[17]	R	8	dod
768	F	3,8	158	ALL9	50	62	63	6	2	0	32	25	neg	47	25	12	neg	neg	Pro-/pre	IM	UNKN	wt	46,XX[28]	CCR	59	alive
1948	M	13,7	UNKN	ALL8	98	99	98	60	30	0	0	0	ND	91	97	20	neg	neg	Cortical	Pre-αβ	UNKN	wt	46,XY,?der(9)(p),add(12)(p12)[21]	CCR	164	alive
1953	M	8,5	130	ALL8	92	10	77	0	92	0	0	0	ND	97	0	12	neg	neg	Pro-/pre	Pre-αβ	UNKN	mutated	47,XY,+8	CCR	152	alive
1959	F	4,3	5,3	ALL8	98	96	96	0	0	0	0	0	ND	6	35	93	αβ	neg	Mature	TCRαβ	UNKN	wt	46,XX,del(14)(q21q31)[4]	CCR	129	alive
2101	M	6,2	167,2	ALL8	95	97	97	29	8	22	0	0	ND	48	36	77	αβ	neg	Mature	TCRαβ	UNKN	wt	47,XY,+?6[6]	R	7	dod
2113	M	3,8	91,5	ALL8	95	95	95	55	93	0	0	0	ND	48	95	13	neg	neg	Cortical	ND	UNKN	mutated	46,XY,t(14;20)(q32;q12)[2]/46,idem,i(9)(q10)[13]/46,idem, del(6)(q13q23),i(9)(q10)[2]/45,idem,-9,i(9)(q10)[2]/87-91, idemx2,-4,-8,-9,i(9)(q10)	CCR	117	alive
2116	F	1,9	387	ALL8	94	94	95	86	1	0	0	0	ND	87	93	7	neg	neg	Cortical	IM	UNKN	mutated	46,XX[30]	CCR	108	alive
2117	M	9,1	226	ALL8	99	99	99	6	82	0	0	0	ND	98	94	9	neg	neg	Pro-/pre	IM	UNKN	mutated	46,XY,t(11;14)(p15;q11)[3]	CCR	108	alive
2641	M	1,6	50	ALL8	88	80	82	67	78	0	9	8	neg	49	74	7	neg	neg	Cortical	IM	UNKN	wt	UNKN	CCR	134	alive
2749	F	15,9	231	ALL9	97	89	97	51	62	0	2	0	neg	1	1	94	neg	γδ	Cortical	TCRγδ	UNKN	mutated	46,X,del(X)(p21),del(5)(q31?),i(9)(q10),add(10)(p1?)	CCR	66	alive
2782	F	13,2	49,5	ALL8	88	7	95	3	0	0	0	0	neg	91	3	4	neg	neg	Pro-/pre	IM	UNKN	wt	46,XX	CCR	113	alive
2790	M	1,1	529,5	ALL9	62	95	75	0	29	1	25	12	neg	2	1	3	neg	neg	Pro-/pre	IM	UNKN	wt	46,XY[31]	CCR	44	alive

Part of this table has been published.[17,18] M, male; F, female; Age, age in years at diagnosis; WBC, white blood cell count; ALL7/8/9, patients treated according to DCOG protocol ALL7, ALL8 or ALL9; ND, not done; pos, positive; neg, negative; UNKN, unknown; wt, wildtype; R, relapse; CCR, continuous complete remission; FU DFS, follow-up disease-free survival (in months); td, toxic death; dod, died of disease; cytβ, cytoplasmic β; CD, cluster of differentiation; smCD3, surface membrane CD3; TCR, T-cell receptor; values for CD7, CD2, CD5, CD1, CD10, CD34, CD33/13, CD4, CD8, and smCD3 are given in percentages. EGIL class., EGIL classification subgroups (pro-/pre, pro/pre-T-cell subgroup, cortical and mature); TCR class., T-cell receptor classification (IM, immature, preαβ, TCRαβ, TCRγδ); NOTCH1 mutated indicates mutations in the hetrodimerization and/or pest domain[20] or insertion mutations in exon 28.[21]

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6

ERBB2 is not a suitable drug target for pediatric T-cell acute lymphoblastic leukemia

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Submitted

ABSTRACT

ERBB2, a transmembrane tyrosine kinase, is overexpressed in several solid tumors such as breast cancer and is successfully used in the clinic as target for the drug trastuzumab in these malignancies. In 40% of patients having B-lineage acute lymphoblastic leukemia (ALL), *ERBB2* is expressed on the surface of leukemic blasts. The role of *ERBB2* in pediatric T-ALL patients is unknown. We studied the expression of *ERBB2* in pediatric T-ALL as a possible new target for therapy. Gene expression analysis of 117 patients showed *ERBB2* to be relatively upregulated in *HOX11*-rearranged T-ALL cases. Using RQ-PCR, we could not confirm differences in *ERBB2* levels between *HOX11*$^{+}$ and HOX11^{-} T-ALL patients. Furthermore, the *ERBB2* expression level in the *HOX11*$^{+}$ cell line ALL-SIL was comparable with the levels for the 7 *HOX11*$^{-}$ T-ALL cell lines. Also, the *HOX11*$^{+}$ T-ALL cell line ALL-SIL as well as 7 *HOX11*$^{-}$ T-ALL cell lines showed trastuzumab resistance *in-vitro*. Therefore, we conclude that *ERBB2* is not a suitable target for therapy in pediatric T-ALL.

INTRODUCTION

ERBB2 (*HER2/NEU*), a transmembrane receptor tyrosine kinase related to the epidermal growth factor receptor (*EGFR*) is located on chromosome 17q21.1 and is overexpressed in 25-30% of breast cancers.[1] Targeting the signal transduction pathway in which ERBB2 is involved by using the anti-ERBB2 monoclonal antibody trastuzumab (Herceptin) has become a very successful therapy for breast cancer. *ERBB2* is expressed on the surface of leukemic blasts in about 40% of patients with B-lineage acute lymphoblastic leukemia (ALL).[2] Chevalier *et al* evaluated the incidence of *ERBB2* expression in ALL patients (B-lineage ALL n=87, T-ALL n=13, 41 adults, 59 children) and suggested that the use of trastuzumab might be a therapeutic option for a selected group of poor-risk adult B-lineage ALL.[3] Others did not identify activating kinase domain mutations within *ERBB* gene family members including *ERBB2* in various hematopoietic malignancies including ALL.[4] T-ALL, a malignant disease of thymocytes, accounts for 15% of pediatric ALL cases and comprise a group of patients who are at high risk for therapy failure compared to patients having B-lineage ALL. The role of *ERBB2* in T-ALL is unknown. In the present study we analyzed whether *ERBB2* is a suitable therapeutic target in T-ALL.

METHODS

Patients

Viably frozen diagnostic bone marrow or peripheral blood samples from 117 pediatric T-ALL patients were used from our cell bank or provided by the Dutch Childhood Oncology Group (DCOG; n=45) or the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL; n=72). All patient samples were processed as described previously and contained >90% of leukemic blasts.[5] Different and recurrent cytogenetic abnormalities were identified using FISH and/or RT-PCR including *TAL1* or *LMO2* rearrangements (n=24 and n=9 patients, respectively), *HOX11L2/TLX3* (n=23 patients), *HOX11/TLX1* translocations (n=7 patients), or the *CALM-AF10* translocation (n=3 patients) as described earlier.[6] For all patients, a signed informed consent was acquired according to the declaration of Helsinki and the study was approved by the ethical committee of the Erasmus MC.

Expression profiling analysis

The 117 samples were analyzed for RNA gene expression levels using the Affymetrix U133 plus 2.0 arrays (Affymetrix, Santa-Clara, USA), as described by us earlier.[7] In brief, total RNA was isolated using Trizol as described previously,[5] and the concentration and integrity were checked on the Agilent 2100 Bioanalyzer (Agilent, Amstelveen, the Netherlands) using a RNA 6000 nano assay. RNA processing and hybridization on the array were performed according to the manufacturer's protocols. Probe intensities were normalized using the variance stabilization procedure (Bioconductor package VSN;[8] as conducted in *R* Bio-conductor (version 2.1.1)). In a supervised analysis, significant and differentially expressed probesets within different T-ALL molecular-cytogenetic subgroups (including *TAL1*, *LMO2*, *HOX11*, *HOX11L2* or

CALM-AF10) were defined using the Wilcoxon t-test ($p_{wilcoxon}$) and corrected for multiple testing by calculating the false discovery rate (FDR) for each probeset. For all 4 subgroups, the top-100 most significant probesets representing expressed genes with significant *p*-values with a FDR lower than $p<0.05$ were selected. This resulted in an overall dataset comprising 590 probesets representing 504 genes (Supplementary Table 1). The Biowisdom Omniviz package (Biowisdom, inc) was used to visualize clustering of T-ALL patients. A Pearson's correlation visualization was calculated using the 590 probesets. The 590 probesets were loaded into the Ingenuity Pathways Analysis program (IPA, Ingenuity systems Inc.). The genes encoded by the probesets were used to generate interaction networks with biological relationships based on manually selected literature references present in the database.

Real-time quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR)

RQ-PCR was used for independent validation of the observed expression in the *HOX11* cluster. Expression levels of *ERBB2* were quantified relative to the expression level of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using an ABI 7700 sequence detection system (PE Applied Biosystems, Fostercity CA, USA) as previously described.[5,9] For detection of *ERBB2* expression levels, the forward primer 5'-CCT-TGC-CCC-ATC-AAC-T-3' and reverse primer 5'-TCT-CCG-CAT-CGT-GTA-CTT-3' were used and this was visualized by SYBR green incorporation during the reaction. The Mann-Whitney *U*-test (MWU) was used to test differences in *ERBB2* mRNA expression levels between $HOX11^+$ versus $HOX11^-$ T-ALL cases (p_{MWU}).

MTT-assay

In-vitro trastuzumab cytotoxicity was determined using the MTT assay as previously described.[10] Cells were incubated in duplicate in the presence of trastuzumab concentrations ranging between 0.000256 to 12500 µg/mL.

RESULTS

In a supervised analysis, differentially expressed probesets that had a FDR rate lower than $p=0.05$ were calculated for various T-ALL patient samples characterized by one of the recurrent molecular-cytogenetic rearrangements i.e. *TAL1* versus non-*TAL1* (100 probesets), *LMO2* versus non-*LMO2* (no significant probesets), *HOX11* versus non-*HOX11* (368 probesets), *HOX11L2* versus non-*HOX11L2* (103 probesets), *HOX11*/*HOX11L2* versus non-*HOX11*/*HOX11L2* (99 probesets) and *CALM-AF10* versus non-*CALM-AF10* (no significant probesets). For the *TAL1*, *HOX11* and/or *HOX11L2* subgroups, we obtained a total of 590 significant and differentially expressed probesets with a FDR lower than $p=0.05$ (Supplementary Table 1). Hierarchical clustering of the entire 117 T-ALL patients relative to the normalized expression values is shown in Figure 1 (for clarity, only the probesets present in the most significant Ingenuity pathway analysis network is shown).

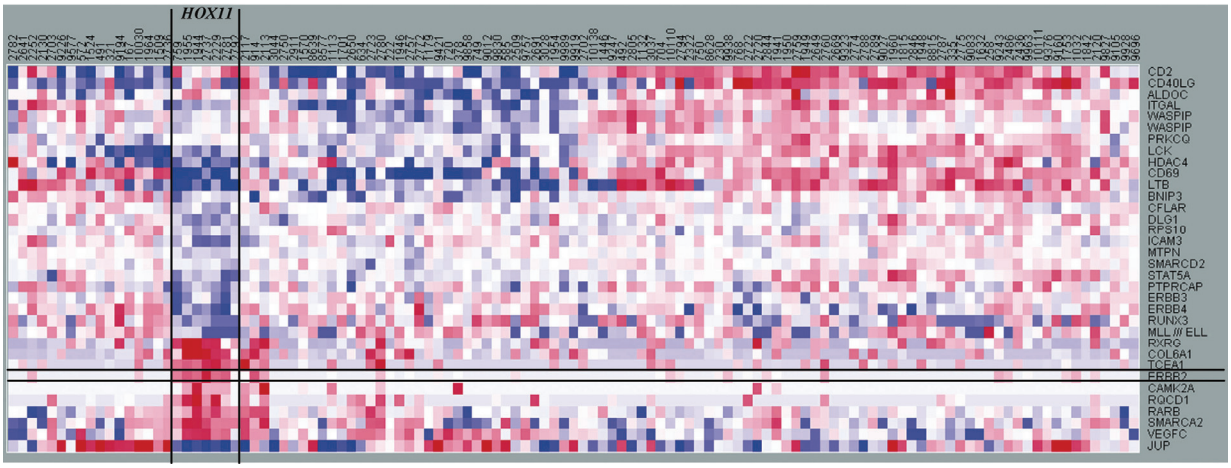

Figure 1. Expression profile (heatmap) showing up-regulation of *ERBB2* in a specific subgroup of pediatric T-ALL patients (for colour figure see page 178)
Patients are shown in columns, genes are presented in rows. Red color indicates that a gene is upregulated, whereas blue indicates that this gene is downregulated. The HOX11-positive T-ALL patients as well as the ERBB2 gene are indicated between lines.

We observed that *ERBB2* was among one of the most significant and differentially expressed probesets in the *HOX11* subgroup ($p_{wilcoxon}$=0.0000379, FDR<0.05). *ERBB2* was expressed at higher levels for *HOX11*-rearranged T-ALL cases compared to other T-ALL cases. The raw *ERBB2* expression level of *HOX11*-positive T-ALL cases was about 10-fold lower than that of *ERBB2*-positive breast cancer patient samples (data not shown; see GEO, breast cancer datasets).

The most significant network (IPA scoring of 56; Figure 2) identified using Ingenuity Pathway Analysis, represents the following top functions: "Cell Death, Gene Expression, Hematological System Development and Function". Interestingly, this network includes *ERBB2* (Figure 2). This suggested that *ERBB2* and associated genes according to literature abstracts were differentially expressed between T-ALL subgroups, and may provide a rationale for increased sensitivity of *HOX11* positive T-ALL cells to trastuzumab.

To validate this observation, the *ERBB2* expression levels were quantified by quantitative PCR analysis (RQ-PCR) in 6 *HOX11*-positive T-ALL cases and compared to the *ERBB2* expression levels in 7 T-ALL cases without *HOX11* rearrangements. In contrast to the microarray data, the relative *ERBB2* expression levels for *HOX11* positive patients measured by PCR did not differ from T-ALL patients lacking *HOX11* abnormalities (p_{MWU} =0.06, Figure 3). In addition, the relative *ERBB2* expression level in the *HOX11*-positive T-ALL cell line ALL-SIL was comparable with the levels for the other 7 *HOX11*-negative T-ALL cell lines (HSB-2, LOUCY, MOLT13, MOLT16, PEER, Pf-382 and SKW3) (Figure 3).

We then determined whether the *in-vitro* sensitivity for trastuzumab for the *HOX11*-positive T-ALL cell line ALL-SIL was higher compared to the other 7 *HOX11*-negative cell lines. All 8 cell lines showed trastuzumab resistance *in-vitro*, including the ALL-SIL cell line (Figure 4).

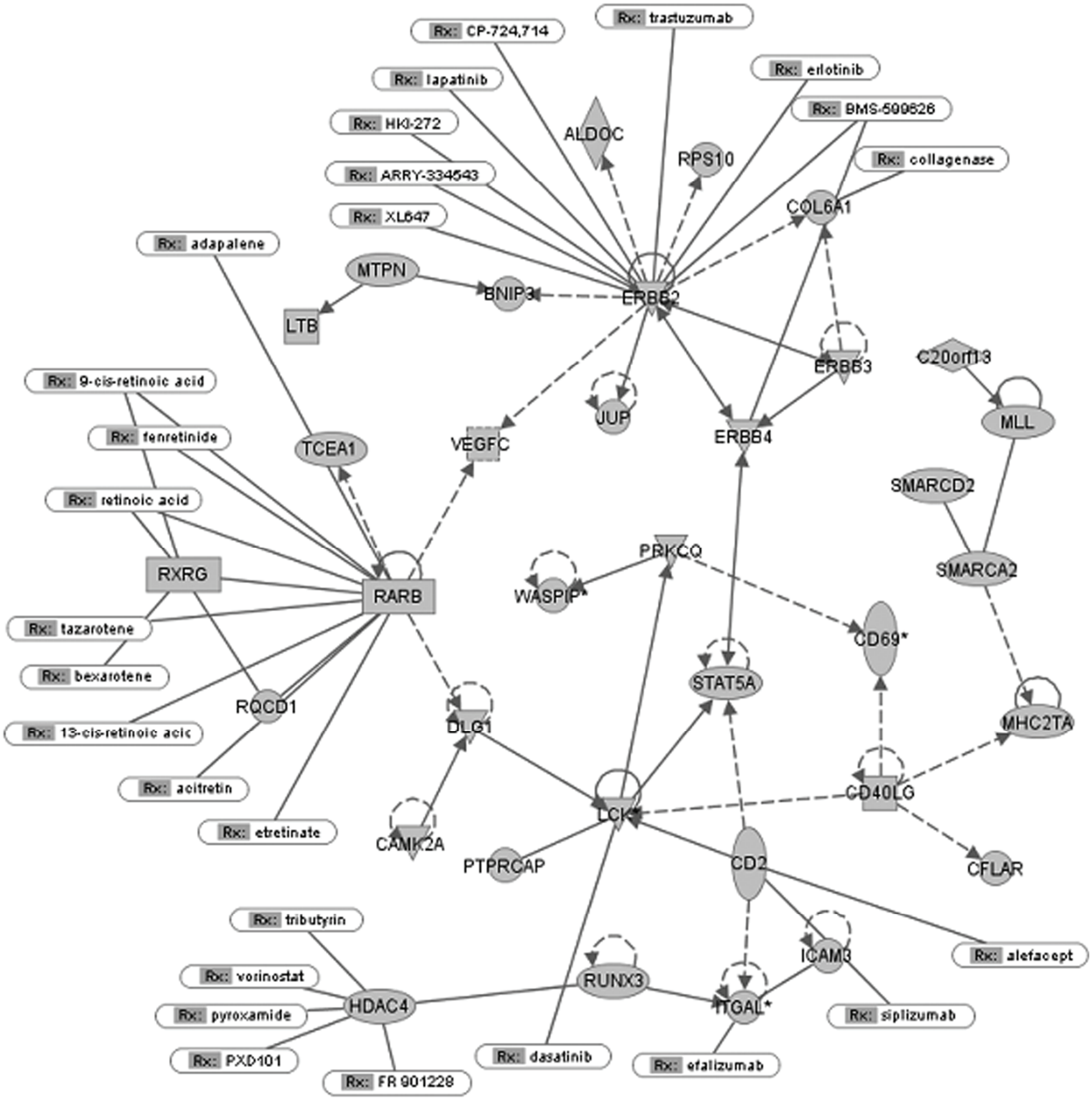

Figure 2. Ingenuity pathways analysis in *HOX11*+ pediatric T-ALL patients
Ingenuity analysis of the 590 probesets revealed the "Cell Death, Gene Expression, Hematological System Development and Function"-pathway as most significant associated pathway. Protein kinases are indicated by triangles; cytokines by squares; enzymes by vertical diamonds; transcription regulators by horizontal ellipses; transmembrane receptors by vertical ellipses; peptidases by horizontal diamonds; translation regulators by hexameres; nuclear receptors by horizontal bars; other kind of proteins by circles; direct protein interactions are indicated by black lines; indirect protein interactions are indicated by dotted lines; drugs directed at specific target proteins are indicated.

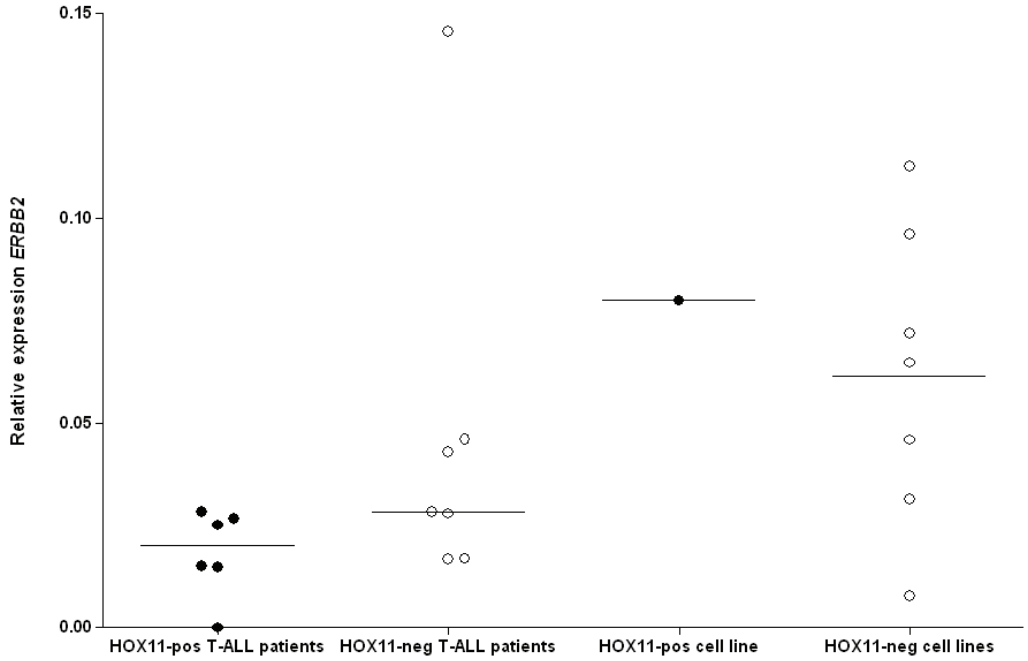

Figure 3. Relative expression of *ERBB2* in *HOX11*$^{+}$ and *HOX11*$^{-}$ pediatric T-ALL patients and in T-ALL cell lines

Patients and cell lines are depicted as rounds. Horizontal lines indicate the median expression levels. HOX11-pos T-ALL patients, HOX11-positive pediatric T-ALL patient samples; HOX11-neg T-ALL patients, HOX11-negative pediatric T-ALL patient samples; HOX11-pos cell line, HOX11-positive pediatric T-ALL cell line ALL-SIL; HOX11-neg cell lines, HOX11-negative pediatric T-ALL cell lines (i.e. HSB-2, LOUCY, MOLT13, MOLT16, PEER, Pf-382 and SKW3).

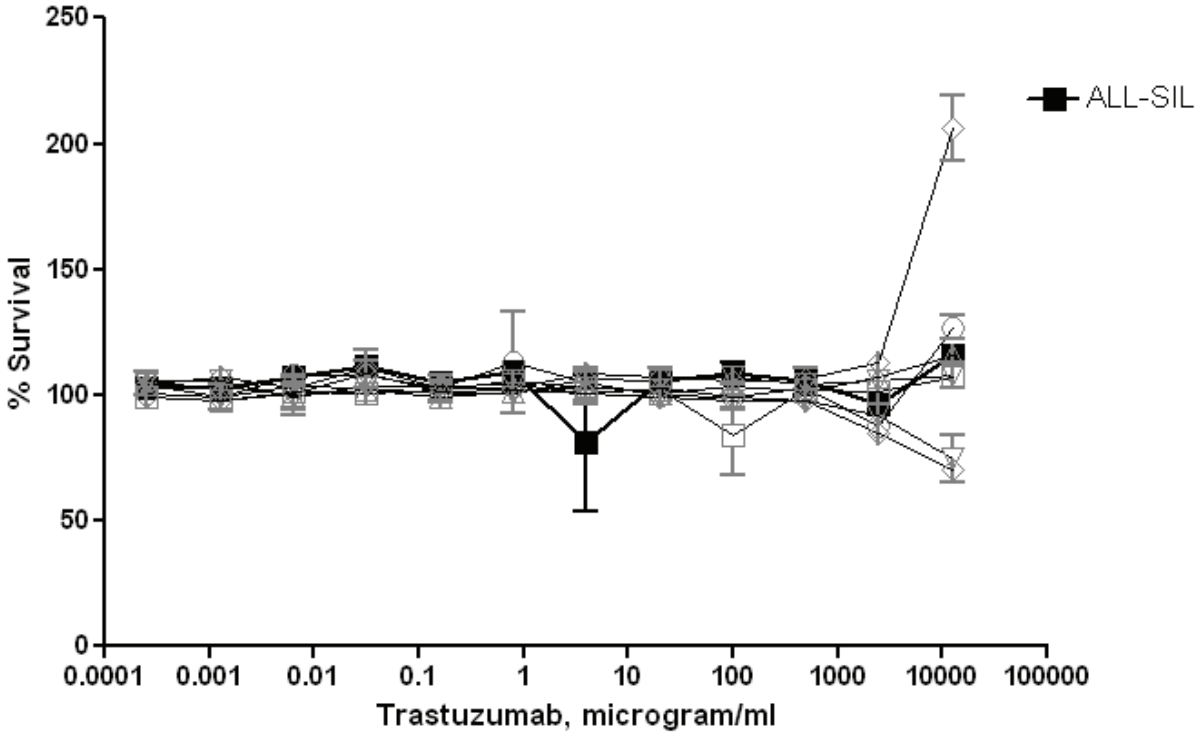

Figure 4. *In-vitro* cytotoxicity of trastuzumab on T-ALL cell lines

Dose response curves of trastuzumab for 8 T-ALL cell lines including one HOX11-positive cell line (ALL-SIL) and 7 HOX11-negative cell lines. Cell survival was measured at 12 concentrations of trastuzumab ranging from 0.00025 µg/mL to 12.500 µg/mL.

DISCUSSION

In about 40% of patients with B-lineage ALL, *ERBB2* is expressed on the surface of leukemic blasts.[2] It has been suggested that target-directed signal transduction inhibition therapy using an anti-*ERBB2* monoclonal antibody might be a therapeutic option for a selected group of poor-risk adult B-lineage ALL.[3] A study by Chevalier *et al* reported surface *ERBB2* expression in 3.4% of children having B-lineage ALL.[3] No studies had been reported on the potential role of *ERBB2* as therapeutic target in pediatric T-ALL patients. In the present study we identified the *ERBB2* gene as one of the most significant and differentially expressed probesets that was expressed at higher levels in *HOX11*-positive T-ALL compared to other genetic subgroups of T-ALL. This finding hinted for increased trastuzumab sensitivity in this T-ALL subgroup. Trastuzumab, a recombinant humanized IgG1 monoclonal antibody directed against ERBB2, is a new and targeted drug with proven high efficacy in breast cancer treatment.[1] Using RQ-PCR, we could however not confirm the relatively high *ERBB2* levels in *HOX11*-positive samples, and demonstrated that *ERBB2* levels were equal to the levels in *HOX11*-negative T-ALL cases. Moreover, in T-ALL cell lines, the *ERBB2* level for the *HOX11*-positive cell line ALL-SIL was equal to that in other T-ALL cell lines and all T-ALL cell lines, including the *HOX11*-positive cell line, were highly resistant to trastuzumab.

We conclude that *ERBB2* is not a suitable target for therapy in *HOX11* positive pediatric T-cell ALL, nor in pediatric T-cell ALL in general.

Supplementary Table 1. List of 590 probesets

Gene	Probe Set ID	Chromosomal Location	Type
MGC4677 /// LOC541471	1552258_at	2p11.2 /// 2q13	HOX11L2
C6orf199	1552299_at	6q21	HOX11
ZNF31	1552499_a_at	1p34.3	HOX11
PCDH10	1552925_at	4q28.3	HOX
ASB10	1553039_a_at	7q36.1	HOX11
BCL2L11	1553096_s_at	2q13	HOX11
PAX1	1553492_a_at	20p11.2	HOX11
GPR6	1553507_a_at	6q21	HOX11
FLJ20674	1553991_s_at	12q24.23	HOX11
ENTPD5	1554094_at	14q24	HOX11
MSRB3	1554126_at	12q14.3	HOX11L2
MSRB3	1554127_s_at	12q14.3	SIL-TAL
PHF21A	1554153_a_at	11p11.2	HOX11
ITGAL	1554240_a_at	16p11.2	HOX
HDAC4	1554322_a_at	2q37.2	HOX11
FBXO7	1554423_a_at	22q12-q13	HOX11
RBM8A	1554602_at	1q12	HOX11
LOC126295	1554628_at	19p13.3	SIL-TAL
C1orf71	1554660_a_at	1q44	HOX11
---	1554887_at	---	HOX11
WNK1	1555068_at	12p13.3	HOX11
ARMC8	1555281_x_at	3q22.3	HOX11
HHIP	1556037_s_at	4q28-q32	HOX/SIL-TAL
CLEC2B	1556209_at	12p13-p12	HOX/HOX11L2
---	1556352_at	---	HOX11
KIAA0478	1556412_at	1pter-q31.3	HOX11
---	1556583_a_at	---	HOX11
---	1556820_a_at	---	SIL-TAL
---	1556821_x_at	---	SIL-TAL
EHBP1L1	1557228_at	11q13.1	HOX11
NOTCH2	1557543_at	1p13-p11	HOX/HOX11L2
---	1557565_a_at	---	HOX11
C14orf145	1557756_a_at	14q31.1	SIL-TAL
LOC143458	1557889_at	11p13	HOX11
UBE2I	1558088_a_at	16p13.3	HOX11
---	1558836_at	---	HOX11L2
---	1558837_a_at	---	HOX11L2
---	1558877_at	---	SIL-TAL
---	1560023_x_at	---	HOX11
GNB5	1560094_at	15q21.2	HOX11
---	1560958_s_at	---	HOX11
TM7SF1	1561195_at	1q42-q43	HOX/SIL-TAL
MUC3B	1561421_a_at	7q22	HOX11
C14orf39	1561985_at	14q23.1	SIL-TAL
---	1562201_x_at	---	SIL-TAL
IL1RAP	1562468_at	3q28	HOX
LOC400548	1562659_at	16q24.1	HOX11
KIAA1295	1562910_at	5q35.1	HOX11
ERBB3	1563253_s_at	12q13	HOX11
---	1563391_at	---	HOX11
KCNT1	1563608_a_at	9q34.3	HOX11
---	1564202_at	1p34.2	HOX11
EMID1	1564251_at	22q12.2	HOX11
MLL /// ELL	1565254_s_at	11q23 /// 19p13.1	HOX11
C8orf8	1565424_at	8p23-p22	HOX11
KIAA0934	1565680_at	10p15.3	HOX11
---	1565705_x_at	---	HOX11
---	1565706_at	---	SIL-TAL
TCEA1	1566208_at	8q11.2	HOX11
---	1566823_a_at	---	HOX11

Gene	Probe Set ID	Chromosomal Location	Type
PER4	1566843_at	7p22.2	HOX11
---	1567173_at	---	HOX11
ABLIM2	1567623_at	4p16-p15	HOX11
GALNT1	1568618_a_at	18q12.1	SIL-TAL
---	1569040_s_at	---	HOX11
---	1569086_at	---	HOX11
---	1569348_at	---	HOX11L2
---	1569537_at	---	HOX11
FLJ90231	1569886_a_at	11q25	SIL-TAL
FAD158	1570007_at	1p22.2	HOX11
CPXM2	1570026_at	10q26.13	HOX11
---	1570087_at	---	HOX/HOX11L2
---	1570269_at	---	HOX11
EIF3S7	200005_at	22q13.1	HOX11
EIF3S5	200023_s_at	11p15.4	HOX11
RPS5	200024_at	19q13.4	HOX11
RPL6	200034_s_at	12q24.1	HOX11
DULLARD	200035_at	17p13	HOX11
SPAG7	200053_at	17p13.2	HOX11
TAF10	200055_at	11p15.3	HOX11
GUK1	200075_s_at	1q32-q41	HOX11/SIL-TAL
ATP6V0B	200078_s_at	1p32.3	HOX11
RPS6	200081_s_at	9p21	HOX11
USP22	200083_at	17p11.2	HOX11
EEF2	200094_s_at	19pter-q12	HOX11
GABARAP	200645_at	17p13.1	HOX11
RPL13A	200715_x_at	19q13.3	HOX11
ANXA5	200782_at	4q26-q28|4q28-q32	SIL-TAL
ODC1	200790_at	2p25	HOX11
HLA-E	200904_at	6p21.3	HOX11
SERPING1	200986_at	11q12-q13.1	HOX/SIL-TAL
SSR4	201004_at	Xq28	HOX11
JUP	201015_s_at	17q21	HOX11
PLD3	201050_at	19q13.2	HOX11
SYNGR2	201079_at	17q25.3	HOX11
PGD	201118_at	1p36.3-p36.13	HOX11
ERP29	201216_at	12q24.13	HOX11
RAD23B	201222_s_at	9q31.2	HOX
ADRBK1	201401_s_at	11q13	HOX11
LRP10	201412_at	14q11.2	HOX
ARF5	201526_at	7q31.3	HOX11
LAMP1	201552_at	13q34	HOX11
IFITM1	201601_x_at	11p15.5	SIL-TAL
CNN2	201605_x_at	21q11.1	HOX
FXR1	201637_s_at	3q28	HOX11
MYBL2	201710_at	20q13.1	SIL-TAL
GALNT1	201722_s_at	18q12.1	SIL-TAL
GALNT1	201723_s_at	18q12.1	SIL-TAL
GALNT1	201724_s_at	18q12.1	SIL-TAL
SMARCD2	201827_at	17q23-q24	HOX11
BNIP3	201848_s_at	10q26.3	HOX
TOMM34	201870_at	---	HOX11
PTPN12	202006_at	7q11.23	SIL-TAL
ALDOC	202022_at	17cen-q12	SIL-TAL
IGBP1	202105_at	Xq13.1-q13.3	HOX11
M6PRBP1	202122_s_at	19p13.3	HOX11
PPP1R2	202165_at	3q29	HOX11
PAM	202336_s_at	5q14-q21	HOX/HOX11L2
NOTCH2	202443_x_at	1p13-p11	HOX
WASPIP	202663_at	2q31.1	HOX/HOX11L2
WASPIP	202665_s_at	2q31.1	HOX/HOX11L2

Gene	Probe Set ID	Chromosomal Location	Type
PKIG	202732_at	20q12-q13.1	HOX/HOX11L2/ SIL-TAL
EBP	202735_at	Xp11.23-p11.22	HOX11
SKIP	202781_s_at	17p13.3	HOX11
FUCA1	202838_at	1p34	HOX
TRAF4	202871_at	17q11-q12	HOX11
PBX2	202875_s_at	6p21.3	HOX11
FHL2	202949_s_at	2q12-q14	HOX11L2
STAT5A	203010_at	17q11.2	HOX11
PTPRK	203038_at	6q22.2-23.1	HOX11L2/SIL-TAL
LAMP2	203041_s_at	Xq24	HOX11
EEF1D	203113_s_at	8q24.3	HOX11
KIAA0040	203143_s_at	1q24-25	HOX11
TRIM14	203147_s_at	9q22.33	HOX11
MAP2K4	203265_s_at	17p11.2	HOX11
TUSC2	203272_s_at	3p21.3	HOX11
SOCS2	203373_at	12q	SIL-TAL
STAM	203544_s_at	10p14-p13	HOX11
RAB4A	203582_s_at	1q42-q43	HOX11
MBNL2	203640_at	13q32.1	HOX/HOX11L2
RREB1	203704_s_at	6p25	HOX11
ITPKB	203723_at	1q42.13	HOX/HOX11L2
PLCB4	203895_at	20p12	SIL-TAL
PLCB4	203896_s_at	20p12	SIL-TAL
ATP5D	203926_x_at	19p13.3	HOX11
SCG2	204035_at	2q35-q36	HOX11
EEF2	204102_s_at	19pter-q12	HOX11
MFNG	204153_s_at	22q12	SIL-TAL
CD37	204192_at	19p13-q13.4	HOX11
PMAIP1	204285_s_at	18q21.32	SIL-TAL
KBTBD11	204301_at	8p23.3	HOX11L2
FLJ10374	204335_at	19p13.3	HOX11
ELMO1	204513_s_at	7p14.1	HOX11
FMNL1	204789_at	17q21	HOX11
TLE4	204872_at	9q21.31	HOX/HOX11L2
LCK	204890_s_at	1p34.3	SIL-TAL
LCK	204891_s_at	1p34.3	SIL-TAL
TOP3A	204946_s_at	17p12-17p11.2|17p12-p11.2	HOX11
ICAM3	204949_at	19p13.3-p13.2	HOX11
PTPRCAP	204960_at	11q13.3	HOX11
NCF1	204961_s_at	7q11.23	HOX11
CLOCK	204980_at	4q12	HOX11
NKRF	205004_at	Xq24	HOX11
ASNS	205047_s_at	7q21.3	HOX11
CIITA	205101_at	16p13	HOX11
TMSL8	205347_s_at	Xq21.33-q22.3	SIL-TAL
DACH1	205471_s_at	13q22	HOX11
RASGRP1	205590_at	15q15	SIL-TAL
IL17R	205707_at	22q11.1	HOX/HOX11L2
GSTM5	205752_s_at	1p13.3	HOX11
CD2	205831_at	1p13	SIL-TAL
GAS2	205848_at	11p14.3-p15.2	HOX11L2
KCNN3	205902_at	1q21.3	HOX11
RXRG	205954_at	1q22-q23	HOX11
GDF10	206159_at	10q11.22	HOX/HOX11L2/ SIL-TAL
IVNS1ABP	206245_s_at	1q25.1-q31.1	HOX11
CPN1	206256_at	10q24.2	HOX11
TAL1	206283_s_at	1p32	SIL-TAL
FLJ10803	206497_at	7p13	HOX11

Gene	Probe Set ID	Chromosomal Location	Type
FLT3	206674_at	13q12	HOX11L2
LILRB5	206856_at	19q13.4	HOX11
ALDH1A2	207016_s_at	15q21.3	SIL-TAL
TLX1	207179_at	10q24	HOX11
SIX6	207250_at	14q22.3-q23	SIL-TAL
CENPF	207331_at	1q32-q41	HOX11
LTB	207339_s_at	6p21.3	HOX11
CAMK2A	207613_s_at	5q32	HOX11
CD40LG	207892_at	Xq26	SIL-TAL
CRHR2	207897_at	7p14.3	HOX11
TRIO	208178_x_at	5p15.1-p14	HOX/HOX11L2
MYH13	208208_at	17p13	HOX11
TLX3	208495_at	5q35.1	HOX/HOX11L2
DDOST	208675_s_at	1p36.1	HOX11
TKT	208699_x_at	3p14.3	HOX11
TKT	208700_s_at	3p14.3	HOX11
OXA1L	208717_at	14q11.2	HOX11
SRP72	208803_s_at	4q11	HOX11
G3BP2	208840_s_at	4q21.1	HOX11
EIF3S4	208887_at	19p13.2	HOX11
UQCRFS1	208909_at	19q12-q13.1	HOX11
TRIO	209011_at	5p15.1-p14	HOX11L2
TRIO	209012_at	5p15.1-p14	HOX/HOX11L2
TRIO	209013_x_at	5p15.1-p14	HOX11L2
UFD1L	209103_s_at	22q11.21	SIL-TAL
MYD88	209124_at	3p22	HOX
MAN2B1	209166_s_at	19cen-q13.1	HOX11
WDR45	209216_at	Xp11.23	HOX11
WDR45	209217_s_at	Xp11.23	HOX11
BNIP2	209308_s_at	15q22.2	HOX11
BCL2L2	209311_at	14q11.2-q12	HOX
RUNX1	209359_x_at	21q22.3	SIL-TAL
STXBP2	209367_at	19p13.3-p13.2	HOX/HOX11L2
EPHX2	209368_at	8p21-p12	SIL-TAL
HLCS	209399_at	21q22.1|21q22.13	HOX11
SPON1	209436_at	11p15.2	HOX11L2
DUSP5	209457_at	10q25	HOX11
CD200	209582_s_at	3q12-q13	HOX/HOX11L2/ SIL-TAL
CD200	209583_s_at	3q12-q13	HOX/SIL-TAL
TRAC	209670_at	14q11	SIL-TAL
TRA@ /// TRAC	209671_x_at	14q11.2 /// 14q11	SIL-TAL
NKX3-1	209706_at	8p21	SIL-TAL
GATA2	209710_at	3q21.3	HOX11
CLEC2B	209732_at	12p13-p12	HOX/HOX11L2
KLK10	209792_s_at	19q13.3-q13.4	HOX11
CD69	209795_at	12p13-p12	HOX/HOX11L2/ HOX11
PRKAA1	209799_at	5p12	HOX11
VEGFC	209946_at	4q34.1-q34.3	HOX
MALT1	210017_at	18q21	SIL-TAL
MALT1	210018_x_at	18q21	SIL-TAL
PCGF1	210022_at	2p13.1	HOX11
APEX1	210027_s_at	14q11.2-q12	HOX11
PRKCQ	210038_at	10p15	SIL-TAL
IDH2	210046_s_at	15q26.1	SIL-TAL
SCN3A	210432_s_at	2q24	HOX
CFLAR	210563_x_at	2q33-q34	HOX11
LST1	210629_x_at	6p21.3	HOX11
DLGAP1	210750_s_at	18p11.3	HOX11
TRBV19 /// TRBC1	210915_x_at	7q34	SIL-TAL

Gene	Probe Set ID	Chromosomal Location	Type
ERBB2	210930_s_at	17q11.2-q12|17q21.1	HOX11
TRA@ /// TRDV2 /// TRAV20 /// TRAJ17 /// TRAC	210972_x_at	14q11.2 /// 14q11	SIL-TAL
CLTB	211043_s_at	4q2-q3|5q35	SIL-TAL
RUNX1	211180_x_at	21q22.3	SIL-TAL
CHRD	211248_s_at	3q27	HOX11
LST1	211581_x_at	6p21.3	HOX11
LST1	211582_x_at	6p21.3	HOX11
RPL3	211666_x_at	22q13	HOX11
TRBV21-1 /// TRBV19 /// TRBV5-4 /// TRBV3-1 /// TRBC1	211796_s_at	7q34	SIL-TAL
TRAF4	211899_s_at	17q11-q12	HOX11
TRA@	211902_x_at	14q11.2	SIL-TAL
EIF4B	211937_at	12q13.13	HOX11
EIF4B	211938_at	12q13.13	HOX11
COL4A1	211981_at	13q34	HOX11
RSL1D1	212018_s_at	16p13.13	HOX11
ATP9A	212062_at	20q13.11-q13.2	SIL-TAL
SLC25A6	212085_at	Xp22.32 and Yp	HOX11
GRINL1A /// Gcom1 /// LOC339970	212241_at	15q22.1 /// 15q21.3 /// 4q13.2	HOX11
CLIPR-59	212358_at	19q13.12	SIL-TAL
NOTCH2	212377_s_at	1p13-p11	HOX
LASS6	212442_s_at	2q24.3	HOX/HOX11
LASS6	212446_s_at	2q24.3	HOX/HOX11
---	212528_at	---	HOX11
ARTS-1	212580_at	5q15	HOX11
KIAA0286	212621_at	12q13.3	HOX11
SEC6L1	212630_at	5p15.33	HOX11
OBSL1	212776_s_at	2q35	HOX11L2
FTL	212788_x_at	19q13.3-q13.4	HOX11
SLC25A6	212826_s_at	Xp22.32 and Yp	HOX11
COL6A1	212940_at	21q22.3	HOX11
PAM	212958_x_at	5q14-q21	HOX
SNAI2	213139_at	8q11	HOX11L2
FTL	213187_x_at	19q13.3-q13.4	HOX11
PPM1B	213225_at	2p21	HOX11
GARNL4	213280_at	17p13.3	HOX/SIL-TAL
LOC155060	213367_at	7q36.1	HOX11
LOC126208	213402_at	19q13.43	HOX11
MFHAS1	213457_at	8p23.1	HOX/HOX11L2/ SIL-TAL
ITGAL	213475_s_at	16p11.2	HOX/SIL-TAL
KIAA1026	213478_at	1p36.21	HOX/HOX11
LOC283768 /// LOC388080 /// LOC388189 /// LOC390535 /// LOC400304 /// LOC440234 /// DKFZp434P162	213737_x_at	15q13.1 /// 15q11.2 /// 15p13	SIL-TAL
CFH	213800_at	1q32	HOX11L2
LGR5	213880_at	12q22-q23	HOX11
KIAA1005	213959_s_at	16q12.2	HOX11
IFITM1	214022_s_at	11p15.5	SIL-TAL
ERBB4	214053_at	2q33.3-q34	HOX11
PPP2R5C	214083_at	14q32	HOX11
LST1	214181_x_at	6p21.3	HOX11
KIAA0368	214356_s_at	9q31.3	SIL-TAL
ADAMTS2	214454_at	5qter	HOX11
ODF1	214485_at	8q22.3	HOX11
LST1	214574_x_at	6p21.3	HOX11
PAM	214620_x_at	5q14-q21	HOX/HOX11L2
TLE4	214688_at	9q21.31	HOX/HOX11L2
CUTL1	214743_at	7q22.1	SIL-TAL
OIP106	214924_s_at	3p25.3-p24.1	SIL-TAL
FEZ2	215000_s_at	2p21	HOX

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DNM1	215116_s_at	9q34	HOX11L2
BTBD9	215261_at	6p21	HOX11
OS9	215399_s_at	12q13	HOX11
LST1	215633_x_at	6p21.3	HOX11
ATP2B3	215911_x_at	Xq28	HOX11
LPXN	216250_s_at	11q12.1	HOX11L2
CLTA	216293_at	9p13	HOX11
KIAA0690	216360_x_at	10q24.1	HOX11
LOC391020	216565_x_at	1p36.11	SIL-TAL
TAL1	216925_s_at	1p32	SIL-TAL
TLE4	216997_x_at	9q21.31	HOX/HOX11L2
RARB	217020_at	3p24	HOX11
MUC3A	217117_x_at	7q22	HOX11
DLG1	217208_s_at	3q29	HOX11
---	217379_at	---	HOX11
EIF3S6IP	217719_at	22q	HOX11
TMBIM1	217730_at	2p24.3-p24.1	HOX11
RPS9	217747_s_at	19q13.4	HOX11
UFC1	217797_at	1q23.3	HOX11
QARS	217846_at	3p21.3-p21.1	HOX11
GTF3C5	217876_at	9q34	HOX11
KCTD3	217894_at	1q41	HOX/HOX11L2
C6orf106	217924_at	6p21.31	HOX11
TNRC5	217931_at	6pter-p12.1	HOX11
C11orf2	217969_at	11q13	HOX11
DHRS8	217989_at	4q22.1	HOX11L2
SLC38A1	218237_s_at	12q13.11	HOX/HOX11L2
C20orf11	218448_at	20q13.33	HOX11
CECR5	218592_s_at	---	HOX/HOX11L2/ SIL-TAL
ZDHHC7	218606_at	16q24.1	HOX11
FLJ12436	218722_s_at	3p21.31	HOX11
RGC32	218723_s_at	13q14.11	HOX11
DKFZp762E1312	218726_at	2q37.1	SIL-TAL
SIGIRR	218921_at	11p15.5	HOX11
CHST12	218927_s_at	7p22	HOX11L2
FLJ11273	218930_s_at	7p21.3	HOX/HOX11L2
CGI-14	219082_at	16p13.3	HOX11
SRR	219205_at	17p13	HOX11
GALNT14	219271_at	2p23.1	HOX11L2
MDS032	219348_at	19p13.11	HOX11
TASP1	219443_at	20p12.1	HOX11L2
PAK6	219461_at	15q14	SIL-TAL
FLJ22624	219544_at	13q22.1	SIL-TAL
DECR2	219664_s_at	16p13.3	HOX11
SMPD3	219695_at	16q22.1	HOX
PLXDC1	219700_at	17q21.1	SIL-TAL
MNS1	219703_at	15q21.3	HOX/HOX11L2
PRG-3	219732_at	9q31.1	HOX11
MGC4093	219766_at	19q13.2	HOX11
C10orf118	219844_at	10q25.3	HOX11
GON4	219846_at	1q22	HOX11
LRRC2	219949_at	3p21.31	SIL-TAL
FLJ21986	220032_at	7q31.31	HOX11
FLJ20674	220137_at	12q24.23	HOX11
CAMK1D	220246_at	10p13	HOX11
MGC5566	220449_at	20q13.12	SIL-TAL
ZSCAN2	220516_at	15q25.2	HOX11
UBXD1	220757_s_at	19p13	HOX11
THEG	220808_at	19pter-p13	HOX11
---	220857_at	---	HOX11

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C14orf162	220887_at	14q24.1	HOX11
MANSC1	220945_x_at	12p13.2	HOX11
COTL1	221059_s_at	16q24.1	HOX11
HRASLS2	221122_at	11q12.3	HOX11
C1orf21	221272_s_at	1q25	HOX11L2
EIF3S12	221494_x_at	19q13.2	HOX11
RPL31	221593_s_at	2q11.2	HOX11
USP3	221654_s_at	15q22.3	HOX11
C19orf22	221764_at	19p13.3	HOX11
TMEM8	221882_s_at	16p13.3	HOX11
NOL11	221970_s_at	17q24.2	HOX
KLHL24	221986_s_at	3q27.1	HOX11
PAPOLA	222035_s_at	14q32.31	HOX11
RBM8A	222443_s_at	1q12	HOX11
BACE2	222446_s_at	21q22.3	HOX11
TOMM22	222474_s_at	22q12-q13	HOX11
C1orf119	222495_at	1p13.3	HOX11
NMD3	222497_x_at	3q26.1	HOX11
WDR41	222503_s_at	5q13.3	SIL-TAL
ABHD10	222697_s_at	3q13.2	HOX11
CHST12	222786_at	7p22	HOX11L2
FLJ11273	222787_s_at	7p21.3	HOX/HOX11L2
MGC2803	223003_at	19p13.13	HOX11
UBXD1	223012_at	19p13	HOX11
FUNDC2	223042_s_at	Xq28	SIL-TAL
PSAT1	223062_s_at	9q21.2	HOX/HOX11L2
MRPL51	223086_x_at	12p13.3-p13.1	HOX/HOX11L2
C1orf21	223125_s_at	1q25	HOX11L2
C1orf21	223126_s_at	1q25	HOX11L2
FEM1A	223175_s_at	19p13.3	HOX11
SEC11L3	223299_at	18q21.32	HOX11
SPATA11	223318_s_at	19p13.3	HOX11
BRI3	223376_s_at	7q21.3	HOX11
ZNF581	223389_s_at	19q13.42	HOX11
RAD23B	223598_at	9q31.2	HOX
ECRG4	223623_at	2q12.2	HOX11
ANUBL1	223624_at	10q11.21	HOX11
---	223661_at	---	HOX11
DPH5	223671_x_at	1p21.2	HOX11
C7orf20	223811_s_at	7p22.3	HOX11
TNFRSF18	223851_s_at	1p36.3	HOX11L2
KCNK17	224049_at	6p21.1	HOX11L2
DPH5	224060_s_at	1p21.2	HOX11
WHSC1L1	224077_at	8p11.2	HOX11
BEX2	224367_at	Xq22	SIL-TAL
TRPM6	224412_s_at	9q21.13	HOX11
RNF7	224439_x_at	3q22-q24	HOX11
TNFRSF18	224553_s_at	1p36.3	HOX11L2
MGC71993	224573_at	17p13.1	HOX11
SLC38A1	224579_at	12q13.11	HOX/HOX11L2
SLC38A1	224580_at	12q13.11	HOX11L2
COTL1	224583_at	16q24.1	HOX11
SLC44A2	224609_at	19p13.1	HOX11
MTPN	224656_s_at	7q33	HOX11
C20orf35	224668_at	20q13.12	HOX11
SNX12	224684_at	Xq13.1	HOX11
NDFIP2	224799_at	13q31.1	HOX/HOX11L2
NDFIP2	224801_at	13q31.1	HOX/HOX11L2
NDFIP2	224802_at	13q31.1	HOX/HOX11L2
SORT1	224818_at	1p21.3-p13.1|1p21.3-p13.1	SIL-TAL

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KIAA1434	224826_at	20p12.3	HOX11
PDRG1	225075_at	20q11.21	HOX11
TMEM32	225125_at	Xq26.3	HOX11
KIAA1718	225142_at	7q34	HOX11
MGC52010	225148_at	22q13.1	HOX11
SLC44A2	225175_s_at	19p13.1	HOX11
KIAA1967	225187_at	8p22	HOX11
MGC2560	225208_s_at	15q25.2	HOX11
MGC2560	225210_s_at	15q25.2	HOX11
---	225220_at	---	HOX11
KPNA4	225268_at	3q25.33	HOX11
MFHAS1	225478_at	8p23.1	HOX/HOX11L2/ SIL-TAL
DDX6	225549_at	11q23.3	HOX11
TIGA1	225698_at	5q21-q22	HOX11
PLEKHH1	225727_at	14q24.1	HOX11
RAB11FIP4	225739_at	---	HOX11
MGC4677 /// LOC541471	225799_at	2p11.2 /// 2q13	HOX11L2
PTPMT1	225901_at	11p11.2	HOX11
PCDH18	225977_at	4q31	HOX11
TALDO1 /// HSUP1	226227_x_at	11p15.5-p15.4 /// 20q13.13	HOX11
LRRC45	226253_at	17q25.3	HOX11
---	226282_at	---	HOX/HOX11L2
ALS2CR13	226431_at	2q33.2	HOX11
FLJ11273	226529_at	7p21.3	HOX/HOX11L2
LOC387758	226769_at	11p14.2	HOX11
STXBP5	226794_at	6q24.3	HOX11L2
ARHGAP9	226906_s_at	12q14	HOX11
SLC39A11	227046_at	17q24.3-q25.1	HOX11
NOTCH2NL	227067_x_at	1q21.2	HOX/HOX11L2
---	227069_at	---	SIL-TAL
SGEF	227197_at	3q25.2	HOX11
---	227252_at	---	HOX/HOX11L2
LOC51136	227268_at	17q23.2	HOX11
LRRC28	227423_at	15q26.3	SIL-TAL
C6orf136	227455_at	6p21.33	HOX11
LOC197322	227464_at	16q24.3	SIL-TAL
---	227579_at	---	HOX11
C10orf118	227701_at	10q25.3	HOX
TRIM59	227801_at	3q25.33	HOX/HOX11L2
SPATA11	227878_s_at	19p13.3	HOX11
FLJ20758	228020_at	2p11.2	HOX11
LOC152485	228046_at	4q31.21-q31.22	SIL-TAL
---	228088_at	---	HOX11
ZNF498	228138_at	7q22.1	HOX11
HAS3	228179_at	16q22.1	HOX11
DISP1	228184_at	1q41	HOX/HOX11L2
UNC13D	228352_at	17q25.1	HOX11
TNRC5	228369_at	6pter-p12.1	HOX11
NBLA10383	228482_at	17p12	SIL-TAL
PCDH10	228635_at	4q28.3	HOX/HOX11L2
MGC33692	228678_at	---	SIL-TAL
LOC150166	228685_at	22q11.21	HOX/HOX11L2
CLK4	228751_at	5q35	HOX11
C9orf81	228774_at	9q21.2	HOX11L2
BVES	228783_at	6q21	HOX11
TUB	228883_at	11p15.5	HOX11
SMARCA2	228926_s_at	9p22.3	HOX11
LOC150166	229101_at	22q11.21	HOX11L2
KIAA1026	229144_at	1p36.21	HOX/SIL-TAL

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EPB41L5	229292_at	2q14.2	HOX11
LOC150166	229295_at	22q11.21	HOX11L2
L3MBTL3	229393_at	6q23	HOX
---	229433_at	---	HOX11
PGM2L1	229553_at	11q13.4	HOX/HOX11L2
---	229573_at	---	HOX11
FLJ40629	229610_at	2q13	SIL-TAL
IRX3	229638_at	16q12.2	HOX/HOX11L2
---	230159_at	---	HOX11
SGOL2	230165_at	2q33.1	HOX11
SCGB3A1	230378_at	5q35-qter	HOX11L2
SLC11A1	230382_at	2q35	HOX11
---	230383_x_at	---	HOX/HOX11L2
SHB	230459_s_at	9p12-p11	HOX11
TMEM46	230493_at	13q12.13	SIL-TAL
CHES1	230515_at	14q24.3-q32.11	HOX11
ZNF233	230919_at	19q13.31	HOX11
---	230953_at	---	HOX11
WASPIP	231182_at	2q31.1	HOX/HOX11L2
C14orf73	231209_at	14q32.32	HOX11
---	231238_at	---	HOX11
RNF133	231373_at	7q31.32	HOX11
---	231403_at	---	HOX11L2
LRRC2	231781_s_at	3p21.31	SIL-TAL
KLK4	231782_s_at	19q13.41	HOX11
CGI-07	231870_s_at	3q26.1	HOX11
PCDHB16	232099_at	5q31	HOX11
MBNL2	232138_at	13q32.1	HOX/HOX11L2
MBNL2	232369_at	13q32.1	HOX/HOX11L2
C14orf145	232635_at	14q31.1	SIL-TAL
UCKL1	232727_at	20q13.33	HOX11
---	232887_at	---	HOX11
MBNL2	232978_at	13q32.1	HOX/HOX11L2
DSCR3	233108_at	21q22.2	HOX11
---	233485_at	---	HOX11
TLE4	233575_s_at	9q21.31	HOX/HOX11L2
TBX18	233889_at	6q14-q15	HOX11
WFDC10A	233913_at	20q13.12	HOX11
SNRPN	234163_at	15q11.2	HOX11
GLTSCR2	234339_s_at	19q13.3	HOX11
LOC400340 /// LOC400353	234423_x_at	15q13.2 /// 15q13.3	SIL-TAL
C20orf86	234511_at	20q13.32	HOX11
C20orf66	234798_x_at	20q13.32	HOX11
ORF1	234806_at	18q23	SIL-TAL
RUNX3	234928_x_at	1p36	HOX11
PLCXD2	235230_at	3q13.2	HOX
NUDT19	235384_at	19q13.11	SIL-TAL
LASS6	235463_s_at	2q24.3	HOX/HOX11
MCEMP1	235568_at	19p13.2	HOX11
SMAD2	235598_at	18q21.1	HOX11
---	235664_at	---	HOX11
---	235705_at	---	HOX11L2
TLE4	235765_at	9q21.31	HOX/HOX11L2
---	235907_at	---	HOX11
FAM80A	235948_at	1p34.2	HOX11
---	235975_at	---	HOX11
ALS2CR3	235983_at	2q33	HOX/HOX11L2
MBNL2	236067_at	13q32.1	HOX/HOX11L2
---	236169_at	---	HOX11
GNAQ	236238_at	9q21	HOX11

Gene	Probe Set ID	Chromosomal Location	Type
BACH2	236307_at	6q15	HOX/HOX11L2
---	236632_at	---	HOX11L2
HOXD13	236681_at	2q31.1	HOX11
RQCD1	236684_at	2q35	HOX11
MBNL2	236699_at	13q32.1	HOX/HOX11L2
BACH2	236796_at	6q15	HOX/HOX11L2
---	236894_at	---	HOX11
---	236937_at	---	HOX11
CD69	237009_at	12p13-p12	HOX/HOX11L2
---	237448_at	---	HOX11
---	237458_at	---	HOX11
HHIP	237466_s_at	4q28-q32	SIL-TAL
---	237520_x_at	---	HOX11
---	237665_at	---	HOX
RPS10	237703_at	6p21.31	HOX11
---	237825_x_at	---	HOX11
C14orf29	237974_at	14q22.1	HOX11L2
ACAD8	238175_at	11q25	SIL-TAL
C5orf13	238410_x_at	5q22.1	HOX11
PGM2L1	238417_at	11q13.4	HOX
MGC19764	238430_x_at	17q12	HOX11L2
---	238676_at	---	HOX11
PCDH9	238919_at	13q14.3-q21.1	HOX11
JDP2	239085_at	14q24.3	HOX11
ZNF75A	239204_at	16p13.11	HOX11
SLC40A1	239723_at	2q32	HOX11
---	239907_at	---	HOX/HOX11L2/ SIL-TAL
LOC116143	240416_at	2p14	HOX11
LOC374443	240572_s_at	12p13.31	HOX/HOX11L2
FBXL7	241457_at	5p15.1	HOX11
KCTD3	241903_at	1q41	HOX
---	241924_at	Xq28	HOX11
LOC388565	241964_at	19q13.42	HOX11
ANKRD17	242116_x_at	4q13.3	HOX11
ITCH	242182_x_at	20q11.22-q11.23	HOX11
C9orf12	242207_at	9q21.33-q22.31	HOX11
---	242321_at	---	HOX/HOX11L2
---	242457_at	---	HOX/HOX11L2
TRAF4	242473_at	17q11-q12	HOX11
BMPR1B	242579_at	4q22-q24	HOX11
HCG18	242812_at	6p21.3	HOX11
---	242861_at	---	HOX11
HYDIN	243354_at	16q22.2	HOX11
---	243356_at	---	SIL-TAL
---	243383_at	---	HOX11
SMARCA4	243655_x_at	19p13.2	HOX11
C14orf145	244033_at	14q31.1	SIL-TAL
LOC401494	244050_at	9p21.3	HOX11
---	244533_at	---	HOX
---	244840_x_at	---	HOX11
N4BP1	32069_at	16q12.1	HOX11
SGSH	35626_at	17q25.3	HOX11
ATP6V0C	36994_at	16p13.3	HOX11
SIGIRR	52940_at	11p15.5	HOX11
C19orf22	55705_at	19p13.3	HOX11
SLC35E1	79005_at	19p13.11	HOX11

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7

Prognostic value of genetic abnormalities in pediatric T-cell acute lymphoblastic leukemia

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Submitted

ABSTRACT

T-ALL subtypes are for more than 50% associated with specific molecular-genetic alterations or immunophenotypic markers and differ in overall survival rates. In this review we will discuss the prognostic value of recurrent molecular-cytogenetic markers that are mostly mutual exclusive and that may delineate separate T-ALL subgroups including *TAL1* and *LMO2* as well as their related family members, *CALM-AF10, SET-NUP214*, *MLL*-fusion products, *HOX11*, and *HOX11L2* in pediatric T-cell Acute Lymphoblastic Leukemia. We also discussed other type of *NOTCH1* activation mutations and *AKT* survival pathway mutations. Some of these abnormalities may provide rationales for new treatment modalities in T-ALL that need to be tested.

INTRODUCTION

Acute leukemia is the most common cancer diagnosed in children. About 80% of the acute leukemias diagnosed among children represent acute lymphoblastic leukemia (ALL). The outcome of pediatric ALL has improved over the last decades with current cure rates of approximately 80 percent.[1] T-cell acute lymphoblastic leukemia (T-ALL), a malignant disease of thymocytes, accounts for about 15% percent of pediatric ALL cases. The leukemic cells in T-ALL are thymic cells that escaped normal differentiation and instead remain trapped into specific developmental stages. Characteristically, T-ALL is more frequent in males than females, presents with a higher white blood cell count (WBC), more frequent central nervous system (CNS) involvement and an enlarged thymus at diagnosis.[2-5] Although cure rates for pediatric T-ALL have improved, an overview in 2000 of long-term results in large prospective trials shows that 30%-50% of T-ALL patients relapse within the first 5 years following diagnosis (Table 1).[6-18]

Molecular-cytogenetic analyses of leukemic cells have contributed to our understanding of the pathogenesis and prognosis of ALL.[1,19] For about half of pediatric T-ALL patients, distinct chromosomal aberrations have been detected. Most of these chromosomal abnormalities are translocations[20,21] that frequently occur as consequence of erroneous rearrangements during attempted V(D)J recombination of the T-cell receptor-beta (TRB) or -alpha/delta (TRA/D) genes.[22] Other abnormalities include deletions, amplification or chromosomal inversions. These abnormalities lead to the activation of oncogenes or the production of specific fusion products. Oncogenes that are frequently involved in such abnormalities include basic helix-loop-helix (bHLH) genes (*MYC, TAL1, TAL2, LYL1*, *bHLHB1*), cysteine-rich (LIM-domain) genes (*LMO1, LMO2*) or homeodomain genes (*HOX11/TLX1, HOX11L2/TLX3*, or *HOXA* genes).[23-25] Other recurrent chromosomal abnormalities include the *CALM-AF10* translocation,[26] the *SET-NUP214* fusion as consequence of the del(9)(q34.11q34.13)[27] and *MLL* rearrangements.[20,21] Most of these recurrent chromosomal rearrangements are mutually exclusive and may result in the activation of specific gene signatures as determined by micro-array experiments.[28] Based on these gene signatures, at least 4 different T-ALL subgroups including the *TAL/LMO*, *HOX11*, *HOX11L2* and *HOXA* T-ALL subgroups can be identified.[23,27-29] These subgroups and the underlying cytogenetic abnormalities have been associated with the expression of specific immunophenotypic markers pointing to their developmental arrest at distinct T-cell differentiation stages.[30,31]

T-ALL therefore represents a heterogeneous disease comprising various molecular-cytogenetically characterized subgroups. It is remarkable that our knowledge concerning the prognostic relevance of these subgroups is limited. In this review we will discuss common molecular-cytogenetic markers in relation to outcome and maturation stage in pediatric T-ALL.

Table 1. Comparison of treatment results of the major treatment consortia for pediatric T-cell acute lyphoblastic leukemia patients[6-18]

Author	Group	Year	Protocol	Total n=	5 yrs EFS in %	10 yrs EFS in %	10 yrs OS in %	T-ALL total n=	SR/HR	T-ALL n=	5 yrs EFS in %	10 yrs EFS in %
Conter, V	AIEOP	2000	AIEOP-ALL82	902	56.4	52.7	63.7	110	SR	35	54.8	54.8
									HR	75	29.3	22.7
			AIEOP-ALL87	632	67	62.8	74.7	74	SR	28	77.9	77.9
									HR	46	37.8	35.4
			AIEOP-ALL88	396	67.8	65.1	75.5	54	SR	12	83.3	83.3
									HR	42	57	50.4
			AIEOP-ALL91	1194	70.8	68.5*	77.4*	144	SR	47	67.4	64.6 *
									HR	97	27.5	27.5 *
Schrappe, M	BFM	2000	ALL-BFM81	611	67.5	63.8	76	62	SR	17	94.1	80.9
									HR	45	71.4	68.6
			ALL-BFM83	653	64.3	61.6	70.9	87	SR	17	41.2	41.2
									HR	70	57.4	55.7
			ALL-BFM86	998	72.1	69	77.9	126	SR	27	92.6	92.6
									HR	99	66.3	66.3
			ALL-BFM90	2178	78	75.9*	83.5*	282	SR	77	72.3	69*
									HR	205	57	57*
Gaynon, P	CCG	2000	CCG83-88	3711	65	62	-	313	SR	100	68	65
									HR	213	58	56
			CCG89-95	5121	75	72.1	-	439	SR	139	80	79 *
									HR	300	70	70 *
Harms, D	COALL	2000	COALL-82	133	55.5	48.6	-	14	SR	2	100	100
									HR	12	58.3	50
			COALL-85	305	68.5	65.4	-	50	SR	9	80	80
									HR	41	73.8	73.8
			COALL-89	215	78.5	75	-	17	SR	4	50	50
									HR	13	69.2	69.2
			COALL-92	538	76.9	-	-	76	SR	23	86.3	-
									HR	53	67.7	-
Kamps, W	DCOG	2000	DCOG-ALL7	218	65.3	63.4	76.4	34	SR	7	57.1	57.1
									HR	27	58	53
Kamps, W	DCOG	2002	DCOG-ALL8	467	73	-	83**	56	SR	14	86	-
									HR	42	67	-

Silverman, L	DFCI	2000	DFCI 81-01	289	74	69	75	39	SR	10	80	80
									HR	29	76	76
			DFCI 85-01	220	78	77	81	20	SR	6	83	83
									HR	14	64	64
			DFCI 87-01	369	77	74	84	36	SR	10	80	80
									HR	26	77	77
			DFCI 91-01	377	83	-	88.2**	28	SR	5	100	-
									HR	23	74	-
Vilmer, E	CLCG-EORTC	2000	CLCG 58881	2065	70.9	68.4*	79*	299	SR	80	68.4	63.8 *
									HR	219	62.9	61.7 *
Gustafsson, G	NOPHO	2000	NOPHO 81-86	832	56.5	53.3	-	66	SR	13	46.2	46.2
									HR	53	52.8	52.8
			NOPHO 86-91	885	69.6	67.6	-	71	SR	15	80	80
									HR	56	48.2	46.4
			NOPHO 91-98	1143	77.6	-	-	103	SR	24	74.8	-
									HR	79	56.9	-
Maloney, K	POG	2000	8691/8704	4404	66.6	63.4	-	439	-	439	51	50.2
Pui, C	St. Jude	2000	study 11	358	71.8	69.3	76.5	62	SR	10	40	40
									HR	52	51.9	50
			study 12	188	67.6	61.5	79.5	110	SR	5	60	60
									HR	24	50	45.8
			study13A	165	76.9	-	83**	22	SR	2	50	-
									HR	20	65	-
Tsuchida, M	Tokyo CCS	2000	L84-11	484	71	66.9	75.2	32	SR	9	55.6	44.4
									HR	23	60.9	60.9
			L89-12	418	67.8	65.3	74.6	43	SR	11	72.7	72.7
									HR	32	51.6	48.1
			L92-13	347	63.4	59.7*	78.6*	41	SR	10	77.1	77.1 *
									HR	31	53.6	53.6 *
Eden, O	UK MRC	2000	UKALLVIII	825	57	54	65	58	SR	12	42	42
									HR	46	39	32
			UKALLX	1612	62	60	71	138	SR	27	59	59
									HR	111	45	44
			UKALLX1	2090	63	60*	81*	206	SR	43	51	45 *
									HR	163	51	51 *

*Patients are categorized according to the NCI (National Cancer Institute) risk criteria; SR, standard risk; HR, high risk; NCI SR, age 1-9 and WBC <50x10^9/l; NCI HR, age >9 or WBC >50x10^9/l; EFS, event-free survival; OS, overall survival; n, number; *, EFS or OS only 8 years; ** EFS or OS only 5 years; WBC, white blood cell count.*

CALM-AF10

The t(10;11)(p13;q14-21) translocation has been reported in acute myeloid leukemia (AML), in particular the M0, M1, and M5 FAB subtypes, in lymphoma, undifferentiated ALL and T-ALL.[26,32,33] In this translocation, the *CALM* gene, an *AP-3*-like family member of clathrin assembly proteins at 11q14-21, is coupled to the putative zinc finger transcription factor *AF10/MLLT10* gene at 10p13.[32,34-37] For this translocation, various fusion transcripts have been described, fusing exons 17 or 19 of *CALM* to exons 4 or 5 of *AF10* including the extended LAP/PHD domain (denoted as 5' fusion transcripts). Alternatively, *CALM* fusions to exons 9 or 10 of *AF10* have been identified (denoted as 3' fusion transcripts).[26]

The translocation t(10;11)(p13;q14-21) has been observed in 4-9% of T-ALLs.[26,33] Only in half of these cases, the translocation was detected by conventional karyotypic analysis.[26] The t(10;11) was found in both immunophenotypic immature and mature T-ALL cases that were committed to the γδ-lineage. It was suggested that the presence of this translocation in immature cases is associated with poor outcome[26,31] (Table 2). The gene signature of *CALM-AF10* positive T-ALL is characterized by the aberrant activation of the *HOXA* genes,[29,38] a signature that was also identified in T-ALL cases having other molecular-cytogenetic defects that may be regarded as belonging to the *HOXA* subgroup.

The T-ALL *HOXA* subgroup

Homeobox genes are master transcriptional regulators in early development and play a critical role in the regulation of hematopoietic stem cell survival and proliferation.[39] Several *HOX* genes play a role in thymocyte development, but their expression is transient. Based upon expression analysis by means of micorarrays, a subgroup of T-ALL has been identified with sustained aberrant expression of members of the *HOXA* family (*HOXA5*, *HOXA9, HOXA10* and *HOXA11*).[29] This subgroup was characterized by various different molecular-cytogenetic abnormalities. Among these aberrations was the above mentioned *CALM-AF10* translocation, but it also comprised cases with an *MLL* rearrangement or cases with an inversion on chromosome 7 leading to the rearrangement of the *TCRB* locus as into the *HOXA* gene cluster.[29,38,40] We recently identified a fourth *HOXA* activation mechanism by array-comparative genomic hybridization analysis in T-ALL patients that had a del(9)(p34.11q34.13) resulting in a SET-NUP214 fusion protein. We have shown that SET-NUP214 functions as a transcription factor that can bind in the promoter regions of various *HOXA* genes including *HOXA9* and *HOXA10*. Here, it recruits the Histone H3K79 methyltransferase hDOT1L,[27] in a similar way as described for the *CALM-AF10*, *MLL-AF10* en *MLL-ENL* fusion products.[41-43] Histone H3-K79 methylation may locally trigger further epigenetic chromatine modifications including histone H3-acetylation resulting in the transcriptional activation of the *HOXA* gene cluster.[27] Whether the total *HOXA* subgroup is associated with poor outcome, such as suggested for *CALM-AF10*,[26,31] is currently unknown.

HOX11/TLX1 positive T-ALL

The *HOX11/TLX1*gene, located on chromosome 10q24, is activated by different translocations, including the t(10;14)(q24;q11) and the t(7;10)(q35;q24),[44] resulting in high *HOX11* expression. It has been reported that *HOX11* may occasionally be expressed at low levels in the absence of *HOX11* translocations.[45,46] This may be caused by the quantitative method used, since we[31] and others[47] found a strict correlation between the presence of *HOX11* rearrangements and high *HOX11* expression levels.

Murine *HOX11* is required for spleen development through a mechanism that affects survival of splanchnic precursors.[48,49] Although the exact role of *HOX11* in T-ALL pathogenesis is largely unknown, *HOX11* may interact with protein phosphatases PP2A and PP1 that normally function within a G2-phase checkpoint. Since *HOX11* is not expressed in normal T-cells, dysregulation of the G2-phase checkpoint may cause illegal entrance of mitosis.[50]

Based upon our gene expression profiling study, *HOX11* translocated T-ALL cases may represent a distinct entity in T-ALL[27,29,40] that most frequently expresses the CD1 marker and resembles cortical thymocytes.[28] *HOX11* positive T-ALL has previously been associated with good outcome, both in children[51,52] as well as in adults[53] (Table 2). Based upon genome-wide expression analyses, it has been demonstrated that this CD1 positive T-ALL entity highly expresses genes involved in cell growth and proliferation and has high levels of the glucocorticoid receptor,[28,53] possibly contributing to the increased responsiveness of this subset to chemotherapy and steroids in particular.[54]

HOX11L2/TLX3

HOX11L2 rearrangements have been found in 21-24% of pediatric T-ALL cases.[30,31,51,55] Based upon gene expression profiling data, *HOX11L2* positive patients may represent a distinct T-ALL entity.[27-29]

HOX11L2 is not expressed during normal T-cell development. For most *HOX11L2* positive cases, *HOX11L2* expression is caused by the t(5;14)(q35;q32) juxtaposing *HOX11L2* to the distal region of *BCL11B*, a gene highly expressed during T-cell differentiation.[56,57] Occasionally, alternative rearrangements with the *TCR*α/δ locus (t(5;14)(q32;q11);[58]) or the *CDK6* gene (t(5;7)(q35;q21);[59]) have been described.

High expression of *HOX11L2* has been associated with poor outcome in some studies,[28,31,60] but not in other studies[51,61,62] (Table 2), which may depend on the therapy given. Alternatively, it has been proposed that differences in treatment outcome may be due to differences in the frequency of CD1 positive *HOX11L2* cases between studies,[55,62] that has been identified as a prognostic favorable marker.[63-65] Our previous study could not confirm this suggestion, since the frequency of CD1 positive *HOX11L2* T-ALL patients who relapsed was equal to the frequency of CD1 positive *HOX11L2* T-ALL cases who did not relapse.[31]

As an alternative explanation,[66] it has been suggested that *HOX11L2* may be preferentially associated with episomal copies of a 500kb region that originates from chromosome 9q34 and that results in a *NUP214-ABL1* fusion product and that might be associated with poor outcome.[67] In line with this hypothesis, we have observed that one *HOX11L2*-positive T-ALL case contained a NUP214-ABL1 positive subclone that equaled 5% of all leukemic cells at diagnosis, but that comprised about 80% of all leukemic cells

at relapse.[68] On the other hand, *NUP214-ABL1* fusions have also been observed in *HOX11* positive T-ALL patients in the absence of relapse as well as in a non-relapsed patient that is characterized with the newly identified *SET-NUP214* fusion resulting in elevated *HOXA* levels.[27]

Rearrangements of the basic helix-loop-helix and LIM-domain only gene families

The basic helix-loop-helix gene (bHLH) *TAL1/SCL* is located on chromosome 1p32, and is rearranged in 17-26% of pediatric T-ALL patients.[30,31,69-71] *TAL1* is involved in chromosomal translocations including the t(1;7)(p32;q35) or the t(1;14)(p32;q24), but is more frequently involved in a interstitial deletion on 1p32 creating a fusion between the promoter region of the *SCL interrupting locus (SIL)* gene and the protein coding domain of the *TAL1* gene.[72-75] These different rearrangements result in aberrant activation of the *TAL1* gene. Less frequently, rearrangements of other homologous bHLH genes have been identified including *TAL2* gene located on 9q32, the *bHLHB1* gene on 21q22 and the *LYL1* gene located on 19p13.2.[76-78]

Basic HLH genes have been shown to be involved in early erythroid hematopoiesis, and these proteins participate in a multi-protein complex that consist of various proteins including LDB1, GATA1, and E2A/HEB.[79] This complex also includes members of the LIM-domain only (LMO) family including *LMO2* and *LMO4.*[79] Although the precise oncogenic mechanism of the *bHLH* genes is not known, it has been suggested that aberrant *TAL1* expression may interfere with differentiation and proliferation by inhibiting the transcriptional activity of E2A/HEB.[80-82] Apart from bHLH gene family members, other components of this complex are also frequently rearranged in T-ALL including *LMO2* on 11p13 or *LMO1* on 11p15. Both genes are aberrantly activated in *TCR*-driven translocations.[83] In 2006, we reported an alternative *LMO2* activation mechanism in T-ALL as consequence of the del(11)(p12p13) interstitial deletion that disrupts a negative regulatory element located upstream of the *LMO2* gene resulting in its high activation.[25] Recent work by others further proposed that loss of this negative regulatory domain may also drive ectopic *LMO2* expression in *LMO2* translocations rather than *LMO2* activation due to illegal juxtaposition in the vicinity of the TCR enhancers as consequence of these translocations.[84]

In the gene expression profiling study performed by Sigaux and coworkers, T-ALL cases with *TAL1* or *LMO2* abnormalities clustered into a single T-ALL cluster.[29] So, abnormalities involving *bHLH* genes or *LMO* genes may result in an (nearly) identical expression profile, in contrast to other studies that propose different mechanisms for different members of both gene families.[28,85] Ferrando *et al* (2002) demonstrated that T-ALL cases with an immunophenotype equivalent to early thymocytes highly expressed the *TAL1*-homologous gene *LYL1* in the absence of detectable *LYL1* rearrangements. This T-ALL subset was proposed as a distinct entity that differed from *TAL1* expressing cases.[28] One explanation for this seeming paradox between these studies[28] is that *bHLH* and *LMO* genes are expressed in normal early thymocytes subsets.[80,86] We have shown that immature T-ALL cases highly expressed *LYL1*, *LMO2* and occasionally *LMO1* as a reminder of their immaturity status rather than as a consequence of an oncogenic hit.[25,31,87] We confirmed that *TAL1* and *LMO2* rearranged

cases have a highly similar gene signature.[28] Other T-ALL cases that co-clustered with *TAL1* or *LMO2* based on their expression profiles contained homologous *LMO1* and/or *TAL2* rearrangements. We therefore propose that *bHLH* (*TAL1*, *TAL2*, *bHLHB1*, *LYL1*) or *LMO* (*LMO2*, *LMO1*) gene rearrangements in T-ALL should be considered as a single *TAL/LMO* entity.[27]

The prognostic relevance of the *TAL/LMO* abnormalities remains to be defined. For *TAL1* rearrangements cases, trends towards favorable outcomes and low levels of minimal residual disease at the end of induction therapy have been observed in various studies[31,51,70,88] (Table 2). This same phenomenon was not confirmed for *LMO2* rearranged cases in our studies. We also observed that *TAL1* abnormalities were associated with high white blood cell count (WBC).

Activation mechanisms of the *NOTCH1* pathway in T-ALL

NOTCH1 encodes a member of the NOTCH transmembrane family, which plays a role in the developmental processes of tissues.[89] During transport to the cell surface, *NOTCH* receptors are proteolytically cleaved and generate mature heterodimeric receptors consisting of an extra-cellular domain that through a non-covalent binding is bound to the transmembrane/intracellular subunit. Binding of *NOTCH1* ligands further triggers 2 consecutive proteolytic cleavages catalyzed by metalloproteases and γ-secretases, and translocation of the intracellular *NOTCH1* (ICN) domain to the nucleus. Here, ICN binds to mastermind and the transcription factor CSL (CBF-1, suppressor of hairless, and Lag-1) leading to the transcriptional activation of specific target genes.[90-95]

NOTCH1 is an important regulator in pluripotent hematopoietic progenitors cells that promotes T-cell lineage commitment and the subsequent assembly of pre-T-cell receptors in immature thymocytes.[96-99] Constitutive *NOTCH* signaling in hematopoietic progenitors disrupts both normal T-cell and B-cell development and may lead to T-cell malignancies.[39] In T-ALL, *NOTCH1* may represent one of the most advanced abnormalities that may enable for uncontrolled proliferation and/or inhibition of apoptosis possibly through up-regulation of the target genes *cMYC, HES1, PTCRA, DELTEX1* and others.[100-102] Activating *NOTCH1* mutations have been found in more than half of all T-ALL patients suggesting a central role for aberrant *NOTCH1* signaling in the pathogenesis of T-ALL.[103] *NOTCH1* mutations were identified in T-ALL cases that also contain one of the subgroup delineating abnormalities like *HOX11*, *HOX11L2*, *TAL1* or *CALM-AF10.*[28,103]

Three distinct types of *NOTCH1* mutations have been identified including missense mutations in the heterodimerization domain (HD), non-sense mutations in the PEST-domain and the most recently discovered juxtamembrane expansion (JME) mutations consisting of internal duplications in exon 28 of the NOTCH1 gene.[103,104] HD and JME mutations in *NOTCH1* may facilitate a conformation change that promotes ligand-independent NOTCH1 cleavages by proteases including gamma-secretase. Therefore patients with these mutations are expected to be sensitive to γ-secretase inhibitors (GSI).[103] First attempts for specific *NOTCH1*-directed therapy by GSI-treatment looked promising as GSIs were shown to induce growth arrest and apoptosis in T-ALL cell lines and primary T-ALL cells.[103,105] However, in phase I and II trials this initial enthusiasm was dampened by the fact that GSIs cause severe gastro-intestinal toxicity.[106]

Table 2. Frequencies of genetic abnormalities and 5 years event-free survival (EFS) in pediatric T-ALL[26,28,30,31,45,51-53,55,56,60,70,71,74,103,107-110,113-120]

Genetic abnormality	Gene involved	Author	Journal	Year	n with abnormality	n without abnormality	Frequency (%)	5 yrs EFS (%)	Reference
t(10;11)(p13;q14-21)	*CALM-AF10*						4-9	0-25	
		Asnafi	Blood	2003	12	131	9	25	26
		Asnafi	Blood	2004	2	38	5	NK	30
		van Grotel	Haematologica	2006	3	69	4	0[α]	31
t(10;14)(q24;q11), t(7;10)(q35;q24), del(10q24)	*HOX11*						2-14	75-92	
		Dube	Blood	1986	2	22	9	NK	114
		Heerema	J Clin Oncol	1998	2	101	2	NK	115
		Schneider	Blood	2000	12	189	6	NK	52
		Ballerini	Blood	2002	3	25	11	NK	60
		Ferrando	Cancer cell	2002	8	59	13.6	92	28
		Kees	Leukemia	2003	2/16	60	12.5	83.3[θ]	45
		Asnafi	Blood	2004	1	39	3	NK	30
		Cave	Blood	2004	10	128	7	83[α]	51
		van Grotel	Haematologica	2006	6	66	8	75[α]	31
		Ferrando	Lancet	2004	16	36	31[δ]	88	53
t(5;14)(q35;q32), t(5;14)(q35;q11), t(5;7)(q35;q21)	*HOX11L2*						21-24	20-76	
		Bernard	Leukemia	2001	5	23	22	NK	56
		Ballerini	Blood	2002	6	22	21	20[β]	60
		Berger	Leukemia	2003	47	164	22	NK	55
		Asnafi	Blood	2004	9	31	23	NK	30
		Cave	Blood	2004	35	118	23	76[α]	51
		van Grotel	Haematologica	2006	17	55	24	57[ηα]/34[ζα]	31
t(1;14)(p33;q11), del(1p32), *TAL1r*	*TAL1*						17-26	59-92	
		Carroll	Blood	1990	5	168	3	NK	74
		Schneider	Blood	2000	5	201	2.5	NK	52
		Aplan	Blood	1992	13	57	19	NK	113
		Bash	Blood	1993	48	134	26	59[γ]	70
		Ballerini	Blood	2002	6	22	21	80[β]	60
		Carlotti	Br J of Haem	2002	21	73	22	NK	71
		Asnafi	Blood	2004	9	31	23	NK	30
		Cave	Blood	2004	17	121	17	75[α]	51
		van Grotel	Haematologica	2006	14	58	19	92[α]	31

Activating HD, JM and PEST domain mutations	*NOTCH1*						34-57	35-90	
		Weng	Science	2004	54	42	56[ε]	NK	103
		Breit	Blood	2006	82	75	52	90	110
		Zhu	Clin cancer res	2006	18	35	34	35	109
		van Grotel	Leukemia	2008	40	30	57	65	111
Inactivating mutations	*PTEN*								
		Palomero	Nat Med	2007	6	29	17	NK	112
Inactivating mutations	*FBXW7*						9-31		
		Malyukova	Cancer Res	2007	8	18	31	NK	117
		Maser	Nature	2007	11	27	29[ε]	NK	118
		O'Neil	J Exp Med	2007	7	74	8.6	NK	107
		Thompson	J Exp Med	2007	7	51	12	NK	108
del(9)(p21)	*p15(CDKN2B)/p16(CDKN2A)*								
		Cayuela	Blood	1996	30	10	75	NK	119
		Zhou	Ped Hematol Onc	1997	15	7	68	NK	120

EFS, event-free survival; δ, adults; ε, adults and children; α, 3 years EFS; β, 2 years EFS; γ, 4 years EFS; θ, 7 years EFS; η, DCOG cohort; ζ, COALL cohort, NK, not known; HD, heterodimerization domain; JM, juxtamembrane domain; PEST, proline, glutamic acid, serine and threonine-rich domain.

None-sense mutations in the PEST domain of *NOTCH1* result in C-terminal NOTCH1 truncation.[103] Truncation of the PEST mutations destroys 2 phospho-degron domains, which are normally bound by the E3-ubiquitin ligase FBXW7. FBXW7 primes activated and nuclear NOTCH1 for proteasomal degradation.[107,108] Loss of function mutations in *FBXW7* were recently identified as an alternative *NOTCH1* activation mechanism that is present in 10-20 percent of T-ALL cases.[107,108] *FBXW7* mutations may occur simultaneously with *NOTCH1* HD mutations but have not been observed in combination with PEST mutations. This latter observation may indicate that NOTCH1 and FBXW7 become mutated in a strict order during multistep T-ALL oncogenesis, whereby PEST mutations result in an FBXW7-insensitive NOTCH1 phenotype eliminating the need for additional *FBXW7* mutations.[108] Mutations in *FBXW7* were identified as a novel cellular GSI resistance mechanism,[107] as it results in the stabilization of the half-life of already γ-secretase-cleaved and activated NOTCH1.

Mutation analyses of T-ALL patients has identified *NOTCH1/FBXW7* mutations in more than 65 percent of the patients belonging to all currently known T-ALL subgroups. Zhu *et al* have studied the clinical significance and prognostic value of *NOTCH1* mutations in both adult and pediatric T-ALL patients. Some cases presented with both a HD and a PEST mutation. The presence of these *NOTCH1* mutations was associated with elevated WBC counts at diagnosis and independently linked to a shorter survival time. Adult T-ALL patients with a *NOTCH1* mutation had a poorer outcome, although this was not confirmed for the pediatric patients, possibly due to the relatively good overall response of pediatric T-ALL.[109] Another study comprising 157 pediatric T-ALL patients, who were treated on the ALL-BFM-2000 protocol, showed a significant relation between the presence of NOTCH1 mutations with good early treatment responses and favorable long-term outcome[110] (Table 2). *NOTCH1* mutations were especially observed in T-ALL cases with a cortical immunophenotype. In our study, we did not observe such a relationship and *NOTCH1* mutations were identified in T-ALL cases of all major molecular-cytogenetic subgroups without any restriction to specific developmental stages. *NOTCH1* was not associated with a good or poor outcome.[111]

One of the underlying explanations for the observed differences between these studies may be that other additional abnormalities may have their effect on outcome as well, and that the incidences of these abnormalities differ between these studies. In this respect, inactivation mutations in the phosphatase *PTEN* were recently identified in 10-20 percent of T-ALL cases, resulting in the sustained activation of the PI3K-AKT pathway.[112] The presence of these *PTEN* mutations made cells completely resistant to NOTCH1 inhibitors, regardless of the type of *NOTCH1* mutations that were present in these cells.[112] The relation between the presence of *PTEN* mutations and outcome needs to be established.

CONCLUSIONS AND FUTURE PERSPECTIVES

T-ALL is clinically regarded as a high-risk disease entity for which with current treatment regimens cure rates of approximately 70% are reached in children. For about 30 percent of pediatric T-ALL patients, therapy is unsuccessful resulting in relapse in the first 2 years after initiation of therapy leading to fatal outcome for most relapsed patients.

Over the last 2 decades, many papers have reported on potential prognostic factors in T-ALL, including clinical parameters, immunophenotypic parameters and molecular-cytogenetic parameters. The picture that emerges from these studies is not consistent, and may be hampered by relative small patient cohorts, inclusion of both pediatric and adult patients for whom treatment outcomes are very different, as well as treatment differences between various studies. Also treatment intensification as applied over the years may have clouded the picture, so that prognosticators for older treatment schedules have no longer prognostic relevance for renewed and intensified protocols. Overall, data on potential prognostic genetic factors for pediatric T-ALL patients are scarce.

What has become evident over the last years is that T-ALL is frequently characterized by different recurrent chromosomal alterations. Some of these abnormalities occur in a mutually exclusive manner delineating unique T-ALL entities. Most of these abnormalities are found in specific maturation stages indicating that they may enforce arrest at specific T-cell development stages, as a prerequisite for further cellular transformation triggered by other and additional genetic abnormalities. By performing genome wide expression analyses, it becomes more and more clear that T-ALL cases with different molecular-cytogenetic abnormalities share common disrupted biological pathways. In this scenario, it has been observed by different research groups that T-ALL cases characterized by, for example, *CALM-AF10*, *MLL*-rearrangements, inv(7)(p15q35) or *SET-NUP214* share a unique profile predominantly characterized by elevated levels of members from the *HOXA* gene cluster, that may be denoted as the *HOXA* subgroup. Also for T-ALL cases having rearrangements affecting *bHLH* and/or *LMO* oncogenes, a common expression profile has been described that may be denoted as the *TAL/LMO* subgroup. Analyses of large T-ALL cohorts as a joint initiative of different research groups will clarify whether associations with outcome such as described for instance for *CALM-AF10* positive patients truly exist regardless of the therapy received. Furthermore, it will clarify whether these associations are also applicable for other T-ALL cases having other rearrangements belonging to the same T-ALL subgroup with a common gene expression profile.

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S

Summary, general discussion and perspectives

Samenvatting, algemene discussie en toekomstperspectief

SUMMARY OF THE THESIS

This thesis aimed to identify new prognostic relevant markers in pediatric T-ALL. It focuses on the prognostic relevance of molecular-cytogenetic abnormalities that may outline separate T-ALL entities. These molecular-cytogenetic abnormalities are studied in relation to specific immunophenotypic markers indicative for arrest at particular development stages.

Chapter 1. In this chapter, we studied the prognostic significance of the mutually exclusive recurrent abnormalities affecting the *TAL1*, *HOX11/TLX1*, or *HOX11L2/TLX3* oncogenes or the t(10;11)(p13;q14) that gives rise to the *CALM-AF10* fusion product. Patients treated on protocols ALL-7, -8 and -9 from the Dutch Childhood Oncology Group (DCOG) were included. Findings were validated in a second and independent T-ALL cohort of the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL). We did not find a prognostic significance for *TAL1* or *HOX11* abnormalities. *HOX11L2* was associated with a poor outcome in both cohorts. The *CALM-AF10* abnormality was observed in only 3 T-ALL cases of the DCOG cohort, and all 3 had early relapses and consequently died. Therefore, *CALM-AF10* may predict for poor outcome, although this finding needs to be independently validated. All of these abnormalities as mentioned above were also associated with *NOTCH1* mutations. *NOTCH1* mutations did not have any prognostic impact within these patient cohorts.

In this chapter, we also describe the observed relationships between distinct molecular-cytogenetic defects and T-cell developmental arrest. For instance, *CALM-AF10* translocations were associated with an immature immunophenotype, whereas some *HOX11L2*-positive patients were immunophenotypically immature or had a mature immunophenotype along the TCRγδ- lineage. *HOX11* had previously been associated with cortical T-cell development by others.[1,2] Indeed, our *HOX11* positive cases all expressed the CD1 marker, which is a hall-mark of cortical T-cell development according to the EGIL classification system.[3] In contrast to other studies[1,4-6] we did not observe a favorable outcome for this molecular-cytogenetic subgroup. This may be due to the low number of patients included. *TAL1* abnormalities were associated with cortical or mature T-cell development along the αβ-lineage.

We observed that the oncogenic transcription factors *TAL1*, *LYL1*, *LMO1* and *LMO2*, are frequently expressed in T-ALL, but in many cases no relation exists with the presence of a genetic defect affecting the loci of these genes. *LYL1* was highly expressed in the *CALM-AF10* and *HOX11L2* subgroups that have an immature phenotype, possibly indicating that high *LYL1* expression is related to this development stage rather than reflecting a hidden *LYL1* abnormality.

Chapter 2. For about 40 percent of the T-ALL cases, a characteristic and subgroup delineating rearrangement is unknown. For this reason, we have applied the genome-wide genomic analysis tool, array-CGH, to search for new genomic regions of amplifications and/or deletions in T-ALL patient samples. We identified a new cryptic

abnormality, i.e. the del(11)(p12p13) in 6 pediatric T-ALL patients. This deletion targets the *LMO2* oncogene in 4 out of 6 cases. For del(11)(p12p13) positive patients involving *LMO2* activation, we observed that the proximal *LMO2* promoter is highly activated and hypothesize that this may be due to the deletion of a negative regulatory sequence just upstream of the *LMO2* gene such as recently identified by others. We conclude that abnormalities involving *LMO2* apart from its activation in different chromosomal translocations are more common in pediatric T-ALL (9%) than thus far appreciated. Because *LMO2* abnormalities occur independent from other recurrent and mutually exclusive cytogenetic abnormalities, we propose that *LMO2*-rearranged T-ALL reflects a separate molecular-cytogenetic T-ALL entity.

Chapter 3. We have used the combination of gene expression profiling and array-CGH analyses to detect new and recurrent molecular cytogenetic abnormalities in T-ALL patients. From this and other studies[2,7] it became clear that T-ALL cases with different molecular-cytogenetic abnormalities may have a similar expression profile. For instance, it became clear that patients bearing rearrangements of members of the *TAL1* gene family or the *LMO* gene family have an identical expression profile, and may therefore belong to the same T-ALL subgroup. Another subgroup, denoted the *HOXA* subgroup, comprises T-ALL patients with *CALM-AF10* translocations, *MLL*-rearrangements, and rearrangements of the *HOXA* cluster itself resulting in high activation of *HOXA* genes.[7] Our strategy also led to the identification of the *SET-NUP214* fusion gene. In 3 out of 5 patients, of whom the expression profiles co-clustered with T-ALL samples with high *HOXA* expression but lacked any of the known *HOXA* subgroup abnormalities, we identified a recurrent 9q34 deletion. We demonstrated that this deletion resulted in an identical *SET-NUP214* fusion product fusing exon 7 of the *SET* gene to exon 18 of the *NUP214* gene. By performing functional analyses, we demonstrated that SET-NUP214 supports oncogenesis by binding to the promotor region of *HOXA* gene members, where it recruits the H3-K79 methyl-transferase HDOT1L. This results in methylation of lysine-79 of the histone H3 moiety that triggers other chromatin modifications including histone H3 acetylation. We hypothesize that these chromatin modification result into a local "open-state" of the *HOXA* locus, that enables transcriptional activation of *HOXA* genes. We further demonstrated that *SET-NUP214* contributes to T-ALL pathogenesis, and is able to inhibit T-cell maturation while enforcing cellular proliferation.

Chapter 4. As described in chapter 1, different molecular-cytogenetic abnormalities in T-ALL seem to be associated with specific T-cell developmental arrest stages. In the study as described in this chapter, it was further investigated whether arrest at specific maturation stages could explain the prognostic significance of these molecular-cytogenetic entities. For this purpose, patients were classified into various developmental stages based upon 2 classification systems, i.e. the EGIL classification system according to Bene *et al*,[3] and the T-cell receptor classification system such as more recently developed by McIntyre and coworkers.[8] Both systems allow for the classification of comparable T-cell developmental stages albeit these are based on the expression of different markers, i.e. the pro-/pre-T-cell stage versus the immature

(IM) stage, the cortical stage versus the pre-αβ stage, and the mature versus the TCRαβ and/or TCRγδ stage. About half of the T-ALL cases were assigned to comparable maturation stages. However, none of the T-cell developmental stages according to EGIL or TCR criteria were associated with outcome, in contrast to specific molecular-cytogenetic abnormalities that are associated with arrest at specific developmental stages. For instance, the *HOX11L2* abnormality predicts for poor outcome, and is specifically associated with early T-cell development or T-cell development in the TCR γδ-lineage. Based on the findings in this study, we concluded that differences in outcome between T-ALL cases characterized by specific molecular-cytogenetic abnormalities cannot be attributed to differences in their T-cell maturation stage. This implies that outcome is stronger determined by deregulated signaling pathways as controlled by activated oncogenes. Future research should therefore focus on the identification of such deregulated signaling pathways in T-ALL subgroups, as well as to the therapeutic applications of specific inhibitors of these signaling pathways.

Chapter 5. We identified that the expression marker CD34 predicted for poor outcome in T-ALL. CD34 is a marker that is normally expressed on hematopoietic stem cells that also highly express various multidrug resistance (MDR) genes. We therefore investigated whether expression of CD34 was associated with elevated levels of MDR genes in pediatric T-ALL as a possible explanation for poor treatment outcome. We identified a relationship between the expression of CD34 with *MDR1* and *MRP1*expression levels, but not with *LRP* expression levels. The expression levels of these MDR genes were however not predictive for outcome. We therefore concluded that the association of CD34 with poor outcome in pediatric T-ALL could not be explained by the elevated expression of *MDR1* and/or *MRP1*.

Chapter 6. In our microarray analysis of the overall gene expression analysis, the *ERBB2* gene was significantly higher expressed in *HOX11*-positive T-ALL patient samples. The *ERBB2* gene is frequently overexpressed in breast cancer, and has become a prime target for specific treatment by the ERBB2 inhibitor trastuzumab/herceptin in women with ERBB2 positive breast cancer.[9] Therefore we investigated whether *ERBB2* could also be a therapeutic target in (*HOX11*-positive) T-ALL patients. Using a quantitative RT-PCR strategy, we could not confirm elevated *ERBB2* levels within the *HOX11*-positive pediatric T-ALL cases compared to T-ALL cases harboring other molecular-cytogenetic abnormalities. Furthermore, the cell line ALL-SIL that carries a *HOX11*-translocation did not demonstrate increased sensitivity to trastuzumab compared to *HOX11*-negative T-ALL cell lines. We therefore concluded that *ERBB2* does not represent a suitable target for therapy in pediatric T-cell ALL patients.

Chapter 7. The current knowledge of most recurrent abnormalities in T-ALL, and their potential prognostic implications is being reviewed in this chapter. We have especially focused on abnormalities that occur mutually exclusive in T-ALL, thereby delineating distinct molecular-cytogenetic entities. Over the last years, research of

many groups was focused on the potential prognostic implications of these genetic markers, but most of these studies came to inconsistent conclusions. There may be several reasons for this, and the most obvious explanations are: low patient numbers limiting the power of the studies, combined analyses of pediatric and adult T-ALL patients for whom treatment responses are different and finally differences in treatment schedules.

FUTURE PERSPECTIVES

The application of overall gene expression analyses by means of microarrays has given new insights into T-ALL. These molecular studies produce vast volumes of data. For future research, it is relevant to maintain our current datasets and to continuously update the annotations for the probesets as present on the microarrays. Increased knowledge and additional insight into gene functions in future will provide new perspectives of aberrantly expressed genes as represented by specific probesets within T-ALL subgroups as described in chapter 3 and 6. The fast development of other and novel molecular biological techniques, including array-based SNP analysis and next generation sequencing, techniques to measure (aberrant) epigenetic modifications to the DNA-histone backbone, new techniques that may reveal novel DNA aberration, studies on the protein level (proteomics), as the combined analyses of these data obtained at various levels (i.e. DNA, RNA, protein), will provide further insights into the molecular etiology of T-ALL. Translational molecular medicine depends on cohort studies were this kind of molecular data is combined with clinical, immunophenotypic and/or karyotypic data. Furthermore, data coming from mouse models mimicking the molecular abnormalities such as observed in the various patient subgroups will give further insight in the contribution of these abnormalities in the pathogenesis of T-ALL. In the future, all this allows for better patient stratification, which may form the basis for diagnostics and treatment by evidence based decision-making.

The biggest merit of T-ALL research of the last years may have been the realization that T-ALL may comprise various subgroups, each having distinct oncogenic mechanisms resulting in characteristic expression signatures. So far, T-ALL can be sub-classified in at least 4 subgroups (*TAL/LMO*, *HOX11*, *HOX11L2*, *HOXA*). It will therefore become more and more important to study prognostic significance not only in the context of specific molecular-cytogenetic abnormalities, but especially in the context of these T-ALL subgroups. To overcome bias by small T-ALL patient cohorts, retrospective and prospective analyses in pediatric T-ALL should be investigated as a joint initiative of different study groups.

As described in this thesis, for about 40 percent of the T-ALL cases mutually exclusive genomic abnormalities have not been identified. Further research should focus on this group of patients that may reveal alternative rearrangements of already known oncogenes, rearrangements of homologous relatives belonging to the same gene family or result in the identification of new oncogenes or fusion products. For this patient group, it also needs to be established whether these cases are part of

already known T-ALL subgroups and share a characteristic expression signature, or form a separate T-ALL subgroup based on their expression. In this respect, it is important to note that research based on the overall gene expression profiles have pointed to the existence of an additional and immature T-ALL subgroup.[7]

The specific oncogenic hits in T-ALL will elicit the (in)activation of specific signaling pathways enabling developmental arrest, cellular resistance towards apoptosis and/or uncontrolled cell proliferation. In future, it will be more and more important to explore these oncogenic pathways, and may reveal new therapeutic targets in subgroups of T-ALL. Other therapeutic targets may emerge from investigations that are focused on the synergistic and pathogenic actions of additional types of abnormalities in T-ALL including abnormalities that result in the activation of selfrenewal pathways (including the *NOTCH1* pathway), the activation of survival pathways (including the AKT pathway), the loss of cell cycle control (including loss of *p15/p16* cell cycle regulators), and/or blockage of T-cell differentiation. Based on this knowledge, we may be able to develop new strategies for therapeutic intervention.

SAMENVATTING

Ons afweersysteem bestaat uit vele soorten witte bloedlichaampjes, met elk een eigen functie. Leukemie is een kwaadaardige ziekte van één van de soorten afweercellen. Bij kinderen is acute lymfatische leukemie (ALL) het meest voorkomend en het ontstaat uit de lymfocyten. We onderscheiden B- en T-lymfocyten in het afweersysteem en uit beide typen kan een ALL ontstaan. Bij kinderen ontstaat ALL meestal uit B-lymfocyten. Leukemie die ontstaat uit T-lymfocyten (T-cel acute lymfatische leukemie, ofwel T-ALL) is zeldzamer en komt voor bij 10-15% van alle kinderen met leukemie. In Nederland worden jaarlijks 100-120 kinderen met ALL gediagnosticeerd, waarvan 15-20 kinderen met een T-ALL. Kinderen met een T-ALL presenteren zich anders dan kinderen met andere vormen van acute lymfatische leukemieën. Karakteristiek voor T-ALL is dat het meer voorkomt bij jongens. Typisch is voorts een hoger leukocytenaantal bij diagnose en frequente centraal zenuwstelsel en mediastinale uitbreiding. De afgelopen decennia is de prognose van kinderen met een T-ALL sterk verbeterd. De huidige genezingskans ligt momenteel zo rond de 70%. Leukemie ontstaat als er tijdens de ontwikkeling van vroege, onrijpe lymfocyten specifieke genetische afwijkingen ontstaan met als gevolg een ongecontroleerde en snelle celdeling. In meer dan 50% van de T-ALL-patiënten herkennen we een of meerdere specifieke moleculair-genetische afwijkingen en/of immunofenotypische markers (markers voor het ontwikkelingsstadium van de T-cel). Op basis van deze bekende defecten onderscheiden we zodoende verschillende subtypen van T-ALL, die mogelijk ook verschillen wat betreft overlevingskansen. Enkele veel voorkomende cytogenetische afwijkingen in T-ALL zijn *HOX11*, *HOX11L2, TAL1*, *CALM-AF10*.

Het doel van dit proefschrift was om nieuwe prognostisch relevante genetische afwijkingen bij kinderen met een T-ALL te identificeren. In dit proefschrift zijn de genetische afwijkingen bestudeerd in relatie tot specifieke immunofenotypische markers, die indicatief zijn voor verschillende ontwikkelingsstadia.

Hoofdstuk 1. In dit hoofdstuk hebben we de prognostische significantie van veel voorkomende afwijkingen bestudeerd. Als eerste is gekeken naar de *TAL1*, *HOX11/TLX1*, en *HOX11L2/TLX3* oncogenen en de t(10;11)(p13;q14) die leidt tot het *CALM-AF10* fusieproduct. In deze studie zijn 72 patiënten geïncludeerd die behandeld zijn volgens Stichting KinderOncologie Nederland (SKION) protocollen. De bevindingen zijn gevalideerd in een tweede onafhankelijk T-ALL cohort van 53 patiënten van de German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL). In deze studie is geen prognostische significantie gevonden voor *TAL1* of *HOX11* afwijkingen. Positiviteit voor *HOX11L2* daarentegen was geassocieerd met een slechte overleving in beide cohorten.

De *CALM-AF10* afwijking werd gezien bij slechts drie T-ALL patiënten binnen het SKION cohort en alle drie hadden een vroeg recidief en zijn overleden. De aanwezigheid van *CALM-AF10* voorspelt dus mogelijk een slechte overlevingskans. Deze bevinding moet verder gevalideerd worden in een grotere groep patiënten. Vervolgens is het ontwikkelingsgen *NOTCH1* onderzocht. Afwijkingen (mutaties) in *NOTCH1* zijn beschreven in T-ALL en worden verondersteld een rol te spelen bij de

uitrijpingstop van de leukemiecellen. Alle afwijkingen hierboven beschreven kwamen voor samen met *NOTCH1* mutaties. *NOTCH1* mutaties hadden zelf geen prognostische betekenis.

Ten slotte beschrijven we in dit hoofdstuk de relatie tussen verscheidene genetische afwijkingen en T-cel ontwikkelingsarrest. Binnen de T-cel ontwikkeling onderscheiden we meestal drie stadia: het immature stadium (vroege/onrijpe T-cellen), het corticale stadium en het mature stadium (rijpe T-cellen). *CALM-AF10* translocaties zijn geassocieerd met een immatuur fenotype. Sommige *HOX11L2*-positieve patiënten zijn immunofenotypisch immatuur, of hebben een matuur immunofenotype volgens de T-cel receptor γδ-lijn. *HOX11* was eerder al door anderen geassocieerd met een corticaal T-cel ontwikkelingsstadium.[1,2] Onze *HOX11*-positieve T-ALL-patiënten toonden allen CD1 expressie. CD1 is een marker voor corticale T-cel ontwikkeling volgens het EGIL classificatiesysteem.[3] In tegenstelling tot andere studies,[1,4-6] hebben wij geen betere overleving geconstateerd voor de *HOX11* subgroep. Beperking in onze studie was het lage aantal patiënten. *TAL1* afwijkingen bleken geassocieerd met cortiaal of matuur T-cel ontwikkelingsstadium van de T-cel receptor αβ-lijn. Verder zagen we dat de oncogene transcriptiefactoren *TAL1*, *LYL1*, *LMO1* en *LMO2*, veelvuldig tot expressie komen in T-ALL. In veel gevallen bestaat er echter geen relatie met de aanwezigheid van een genetisch defect dat de plaats van deze genen op de chromosomen beïnvloedt. *LYL1* kwam hoog tot expressie in de *CALM-AF10* en *HOX11L2* subgroepen, die beide een immatuur fenotype hebben. Dit wijst er mogelijk op dat hoge *LYL1* expressie gerelateerd is aan een immatuur ontwikkelingsstadium, in plaats van dat het wijst op een verborgen *LYL1* abnormaliteit.

Hoofdstuk 2. Voor ongeveer 40% van de T-ALL gevallen is er geen specifieke genetische afwijking bekend. Deze patiënten werden bestudeerd met een genoomwijde analysemethode, de array-CGH. Deze techniek maakt het mogelijk het gehele genoom te onderzoeken op genetische afwijkingen (mutaties, deleties, amplificaties van DNA-regio's) om zo niet eerder gekende afwijkingen te identificeren. Bij zes kinderen met een T-ALL hebben we een nieuwe overeenkomstige cryptische afwijking geïdentificeerd, de del(11)(p12p13). Deze deletie van een deel van chromosoom 11 heeft een activerende invloed op het *LMO2* oncogen bij 4 van de 6 patiënten. Met name zagen we dat bij de del(11)(p12p13)-positieve patiënten die een verhoogde activiteit van het LMO2 gen vertoonden, de proximale *LMO2* promotor geactiveerd was. We veronderstelden dat dit het gevolg was van de deletie van een negatieve regulatoire sequentie vlak vóór het *LMO2* gen. Dit was recent ook zo beschreven door anderen. Wij concluderen dat abnormaliteiten die betrekking hebben op LMO2 meer voorkomen bij kinderen met T-ALL (9%) dan tot nu toe aangenomen is. Omdat *LMO2* abnormaliteiten onafhankelijk voorkomen van andere cytogenetische afwijkingen, denken wij dat T-ALL-patiënten met een *LMO2* afwijking een aparte genetische T-ALL entiteit vertegenwoordigen

Hoofdstuk 3. In dit hoofdstuk is gebruik gemaakt van de combinatie van gen expressie profilering en array-CGH analyse van het SKION en COALL T-ALL cohort om moleculair-genetische afwijkingen te classificeren naar expressiepatroon. Deze en

andere studies hebben duidelijk gemaakt dat T-ALL-patiënten met verschillende genetische afwijkingen mogelijk een zelfde genexpressieprofiel hebben. Zo hebben we vastgesteld dat patiënten die een afwijking hadden binnen de *TAL1* genfamilie, of een afwijking binnen de *LMO* genfamilie, een identiek genexpressieprofiel bezaten, en dat ze mogelijk daarom tot dezelfde T-ALL subgroep behoren. Een andere subgroep, de *HOXA* subgroep, die T-ALL-patiënten omvat met *CALM-AF10* translocaties, *MLL*-rearrangements, en rearrangements van het *HOXA* cluster, toont een hoge activatie van *HOXA* genen.[7] In onze studie vertoonden vijf patiënten met een onbekende genetische afwijking een genexpressieprofiel gelijkend op dat van de *HOXA* subgroep. Bij 3 van deze 5 patiënten vonden we een 9q34 deletie, maar geen expressie van één van de bekende *HOXA* subgroep afwijkingen (*CALM-AF10*, *MLL*-rearrangements of *HOXA*-rearrangements). We toonden aan dat deze deletie aanleiding geeft tot een uniek *SET-NUP214* fusieproduct, waarbij exon 7 van het *SET* gen fuseert met exon 18 van het *NUP214* gen. Bindingassays lieten zien dat het SET-NUP214 fusie-eiwit bindt aan de promotor regio van *HOXA* genleden, en vervolgens het H3-K79 methyltransferase HDOT1L rekruteert. Dit resulteert in de methylering van lysine-79 van histon H3, wat andere chromatinemodificaties triggert, inclusief histon H3 acetylering. Deze chromatinemodificatie resulteert in een lokaal 'open-chromatine' van het *HOXA* locus, wat transcriptionele activatie van *HOXA* genen mogelijk maakt. Verder hebben we laten zien dat *SET-NUP214* bijdraagt aan de pathogenese van T-ALL doordat *SET-NUP214* in staat is om T-cell maturatie te remmen, terwijl het cel proliferatie stimuleert.

Hoofdstuk 4. Zoals beschreven in hoofdstuk 1, lijken verschillende genetische afwijkingen geassocieerd met specifieke T-cell ontwikkelings stadia. In deze studie werd onderzocht of een specifiek maturatie arrest de prognostische significantie van genetische entiteiten kon verklaren. Hiervoor werden patiënten in verschillende ontwikkelingsstadia geclassificeerd volgens twee systemen, de EGIL-classificatie volgens Bene[3] en de T-cel-receptorclassificatie zoals die onlangs ontwikkeld is door de groep van McIntyre.[8] Beide systemen veronderstellen de classificatie van vergelijkbare T-cel ontwikkelingsstadia, hoewel deze gebaseerd zijn op de expressie van verschillende markers: het pro-/pre-T-cell stadium versus het immature stadium, het corticale stadium versus het pre-αβ stadium en het mature stadium versus het TCRαβ en/of TCRγδ stadium. Bijna de helft van de T-ALL casussen werden volgens de 2 systemen ingedeeld in een vergelijkbaar maturatiestadium. Echter, in tegenstelling tot enkele genetische afwijkingen die geassocieerd zijn met prognose en met een arrest in een bepaald ontwikkelingsstadium, bleek geen van de T-cel ontwikkelingsstadia volgens EGIL of TCR criteria zelf geassocieerd met de overlevingskans. Bijvoorbeeld, *HOX11L2* voorspelt een slechte overlevingskans en is specifiek geassocieerd met vroege T-cel ontwikkeling van de TCR-γδ-lijn. Gebaseerd op de bevindingen in deze studie hebben we geconcludeerd dat verschillen in overleving tussen kinderen met T-ALL, die gekarakteriseerd worden door specifieke genetische afwijkingen niet te wijten zijn aan verschillen in het T-cel maturatie-stadium. Dit impliceert dat overlevingskans meer bepaald wordt door deregulatie van signaalpaden, die gecontroleerd worden door geactiveerde oncogenen. In de toekomst

zou onderzoek zich moeten richten op de identificatie van deze gedereguleerde signaalpaden binnen T-ALL subgroepen, evenals op specifieke remmers van deze signaalpaden.

Hoofdstuk 5. Uit eerdere analyses is gebleken dat CD34 een marker is voor een lage overlevingskans bij T-ALL. CD34 is een marker die normaal tot expressie komt op hematopoïetische stamcellen. Op stamcellen komen ook verschillende multi-drug resistentie (MDR) genen tot expressie. Hoge expressie of activiteit van MDR genen wordt in het algemeen in verband gebracht met een slechtere prognose en, zoals hun naam suggereert, resistentie tegen cytostatica. Daarom hebben we onderzocht of CD34 geassocieerd was met verhoogde expressieniveaus van MDR genen bij kinderen met een T-ALL. We vonden een associatie tussen de expressie van CD34 en die van *MDR1* en *MRP1*, maar niet van *LRP*. De hoge expressie van deze MDR genen bleek echter niet voorspellend voor overleving. We concluderen daarom dat de associatie van CD34 met slechte overleving bij kinderen met een T-ALL niet verklaard kan worden door de verhoogde expressie van *MDR1* en/of *MRP1*.

Hoofdstuk 6. In onze genoom brede genexpressie-analyse, kwam het *ERBB2* gen significant hoger tot expressie bij *HOX11* positieve T-ALL-patiënten. Het *ERBB2* gen komt frequent tot overexpressie bij borstkanker en is een belangrijk doelwit geworden voor specifieke behandeling met de *ERBB2* inhibitor trastuzumab (herceptin®) voor vrouwen met een *ERBB2* positieve borstkanker.[9] Daarom hebben we onderzocht of *ERBB2* zou kunnen fungeren als therapeutisch doelwit bij (*HOX11*-positieve) T-ALL-patiënten. Gebruikmakend van kwantitatieve RT-PCR strategie, konden we echter geen verhoogde *ERBB2* levels bij kinderen met *HOX11*-positieve T-ALL bevestigen, in vergelijking met T-ALL-patiënten met een andere moleculair-cytogenetische afwijking. Daarnaast liet de *HOX11*-positieve cellijn ALL-SIL geen verhoogde gevoeligheid voor trastuzumab zien, in vergelijking met *HOX11* negatieve T-ALL cellijnen. Daarom hebben we geconcludeerd dat *ERBB2* geen bruikbaar target is voor therapie bij kinderen met een T-cel leukemie.

Hoofdstuk 7. De huidige kennis van de meest voorkomende afwijkingen in T-ALL en hun potentiële prognostische implicaties worden samengevat in dit hoofdstuk. De nadruk in dit hoofdstuk ligt op afwijkingen die het meest voorkomen in T-ALL, en daarmee verschillende genetische entiteiten vertegenwoordigen. In de afgelopen jaren, heeft het onderzoek van veel groepen zich gericht op het mogelijke prognostische belang van deze genetische markers. De meeste van deze studies komen tot tegenstrijdige conclusies. De meest voor de hand liggende verklaringen hiervoor zijn, dat de meeste studies een beperkt aantal patiënten bevatten hetgeen de analytische kracht van de studies vermindert. Andere oorzaken zijn dat in veel studies kinderen en volwassenen met T-ALL samen worden geanalyseerd, terwijl de overleving van die groepen verschillend is en het dus mogelijk twee verschillende subgroepen betreft. Ten slotte zijn er tussen de verschillende studies belangrijke verschillen in therapie die kunnen bijdragen aan de uitkomst van de patiënten en dus ook van prognostische analyses.

TOEKOMSTPERSPECTIEF

Dit proefschrift heeft laten zien dat het uitvoeren van zogenaamde high-throughput analyses op patiëntencohorten nieuwe inzichten kunnen geven over bestaande afwijkingen en ook nieuwe afwijkingen kan opsporen. De snelle ontwikkeling van bestaande en nieuwe moleculair-biologische technieken, waaronder array-gebaseerde SNP analyse, next generation sequencing, technieken om epigenetische modificaties te meten van het DNA-histon skelet, nieuwe technieken die nieuwe DNA-afwijkingen aan het licht zullen brengen, studies op eiwitniveau (proteomics), evenals de gecombineerde analyses van deze data die op verschillende niveaus verkregen is (DNA, RNA, eiwit), zal ons in de toekomst meer inzicht geven in de moleculaire etiologie van T-ALL.

Eén van de grote stappen voorwaarts binnen T-ALL onderzoek in de laatste jaren is het inzicht dat er verschillende T-ALL subgroepen bestaan. De subgroepen hebben elk verschillende oncogene mechanismen, die resulteren in karakteristieke expressieprofielen en mogelijk ook patiëntenoverleving. T-ALL kan onderverdeeld worden in tenminste 4 subgroepen (*TAL/LMO*, *HOX11*, *HOX11L2*, *HOXA*). Dit biedt een belangrijk houvast bij studie van de patiëntenoverleving en de context van specifieke cytogenetische afwijkingen. Een ander belangrijke stap in het onderzoek naar T-ALL zou zijn het bundelen van patiëntengroepen uit verschillende T-ALL studiegroepen voor retrospectieve en prospectieve analyses met voldoende statistische power. Nauwere samenwerking is in het belang van patiënten met een zeldzame aandoening.

Bij 40 procent van de T-ALL-patiënten is de genetische afwijking onbekend. Toekomstig onderzoek moet zich richten op deze groep patiënten. Het zal inzicht geven voor de gehele populatie van T-ALL-patiënten omdat er nieuwe genpaden belangrijk zullen blijken te zijn, maar ook dat er alternatieve defecten van bekende paden mogelijk zijn. Hieruit zal ook blijken of deze patiënten bij een reeds bestaande T-ALL subgroep horen, o.a. vanwege eenzelfde genexpressie profiel, of dat ze een aparte T-ALL subgroep vormen, als beschreven in hoofdstuk 3.[7]

Specifieke oncogene afwijkingen binnen T-ALL zullen leiden tot (in)activatie van specifieke signaalpaden die een ontwikkelingsstop, cellulaire medicijn ongevoeligheid of ongecontroleerde celgroei veroorzaken. Het uiteindelijke doel zal zijn een doelgerichte behandeling van individuele patiënten met een T-ALL op basis van hun subgroep-specifieke indeling of op basis van nieuwe, nog te ontwikkelen medicijnen die zich richten tegen subgroep-specifieke defecten.

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CV

Curriculum vitae

Martine van Grotel was born on the 22nd of November 1975 in Asten, a small village in the south of the Netherlands. She finished the Gymnasium in Deurne (St. Willibrord Gymnasium) in 1995. After she graduated at the Academic for Physical Education in Tilburg (1999, Academie voor Lichamelijke Opvoeding), she started studying medicine at the Erasmus University in Rotterdam. To finish her doctoral phase in Spring 2003, she had the opportunity to work on a research project in the Laboratory of Medical Oncology (Nadler Lab, Dr. A.A. Cardoso) at the Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA. In September 2003 she started the PhD research project 'Identification of prognositc genetic factors in pediatric T-cell acute lymphoblastic leukemia' at the Department of Pediatric Oncology/Hematology, Erasmus University Medical Center–Sophia Children's Hospital, Rotterdam (Promotor Prof. Dr. R. Pieters).
Martine van Grotel was one of the data managers of the Dutch Childhood Oncology Group (DCOG; 2003-2007). In April 2008 she finished her study medicine and in May 2008 she started to work in the Erasmus MC – Sophia Children's Hospital.

D

Dankwoord

Hier zit ik dan, op 't dak van het hotel in Barcelona, in de stalende zon. Het is zover, het is af! Alles is geschreven, de figuren zijn gemaakt, de laatste klussen nog en dan kan het naar de drukker. Een heerlijk gevoel. De afgelopen 5 jaar op het lab heb ik als een mooie periode ervaren. Tijd om nu iedereen te bedanken die, op welke wijze dan ook, een bijdrage geleverd heeft aan de totstandkoming van dit proefschrift.

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Leden van de commissie; Prof. dr. Van der Spek, Prof. dr. Van der Heijden, Prof. dr. Cornelissen, Prof. dr. Hoogerbrugge, Dr. Van den Heuvel-Eibrink, Dr. Van Wering hartelijk dank dat u bereid bent deel uit te maken van mijn commissie.

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Professor Van der Spek, beste Peter. Jij en je afdeling hebben me laten zien hoe briljant samenwerking kan zijn. Je hebt me wegwijs gemaakt in de mogelijkheden die bio-informatica te bieden heeft. Je enthousiasme en positiviteit werken aanstekelijk. In de vroege ochtend hebben we vele uren achter je pc doorgebracht, analyserend en discussierend over data en de mogelijke betekenis daarvan. Nieuwe ideeën zijn ontstaan. Dank voor de mogelijkheden en de geboden kansen. 'The land of the rising sun', Japan, het was fantastisch. Ik zal dat niet snel vergeten.

Dr. Beverloo, beste Berna. Je stond aan de wieg van mijn wetenschappelijke carrière. Als groentje kwam ik bij jou op het lab leren pipetteren en gels maken. Dank voor de fijne samenwerking, je collegialiteit en je vriendschap.

Dr. Van Zutven, beste Laura. Dank dat je de tijd hebt genomen om me wegwijs te maken in de wondere wereld van het lab en van chromosomen. Hoe werkt een pipet en waarom maak je een gel en wat kan je daar dan mee? Een slot? In Boston heb ik veel profijt gehad van de technieken die ik van jou en Berna geleerd heb. Veel succes met de opleiding tot klinisch-geneticus.

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Dr. Van den Heuvel-Eibrink, lieve Marry. In de jaren dat ik je ken, ben je niet alleen bezig geweest met de toekomst van kinderen, je eigen toekomst, maar ook met mijn toekomst. Het fantastische van het leven is, dat er momenten zijn dat je iemand ontmoet die je inspireert en laat leven. Alles vanuit vertrouwen, respect, oprechtheid en gewoon vanuit gevoel. Ik ben een bevoorrecht persoon dat ik, uit die miljarden bewoners van deze aarde, jou heb mogen ontmoeten, 'serendipity'? Onze collegialiteit, maar bovenal onze vriendschap betekent zeer veel voor me. Dank voor alles wat je voor me hebt betekend en nog gaat betekenen. Ik hoop in de toekomst nog veel van je te mogen leren. Dank dat je er was en dank dat je er bent. Gerrit-Jan, Simone en Floris dank voor jullie vriendschap en belangstelling.

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Dr. Van Vlierberghe, beste Pieter. Ik denk dat er buiten Jules, jij en ik er weinigen zijn die alle T-ALL sample-nummers met bijbehorende afwijkingen kunnen dromen. Het was prettig om met je samen te werken, dank voor al je hulp. Het ga je goed in België.

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Overige 'roomies'. Lizet, samenwerken voor SKION tijdens de promotie. Ik heb je leren kennen als een harde werker, secuur in hetgeen je doet. Heel veel succes in je promotietraject. Marjolein en Anneloes, verrassend verfrissend zijn jullie voor de kamer. Jullie brengen vrolijkheid, openheid en gezelligheid met je mee. Marjo, heel veel succes met de voortzetting van je project. Nog even en je bent op hetzelfde punt als ik aan gekomen. Ik weet zeker dat je een prima kinderarts wordt. Anneloes, veel succes met je studie geneeskunde. Ook jij gaat een perfecte dokter worden.

Jeanine en Jacqueline, dank jullie wel voor alle zeergewaarde hulp de afgelopen jaren.

Margo Terlouw, hartelijk dank voor het in orde maken van het boekje. Ik ben er erg blij mee.

Geert, Marian, Marloes en Frank. Aan het begin van mijn geneeskundestudie zocht ik een plaats in een herberg, en jullie hebben me die plaats geboden, vijf sterren. Heerlijk was het om deel uit te maken van jullie gezin. Ik had het zo 6 jaar vol kunnen houden en zal mijn leven niet vergeten hoe goed jullie voor me waren en zijn. Ik heb er van genoten en waardeer het tot de dag van vandaag.

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Lieve Edith, Lennard, Eline en Luc. Edith, tussen ons bestaat een hechte vriendschap. Twee handen op één buik, een half woord is genoeg. Hockeyen, carnaval vieren, skiën, een reservewiel dat onder de auto uit valt, het zijn maar enkele momenten uit de rijke jaren vriendschap. Oprecht, eerlijk en nuchter. Samen met Lennard, Eline en Luc leef je je leven. Ik vind het steeds weer bijzonder dat ik daar zo nu en dan deel van uit mag maken. Altijd welkom, altijd tijd, niets is te veel. De warmte die jullie als gezin uitstralen, dat doet me veel. Dank je dat je mijn paranimf wil zijn.

Lieve pa en ma. Al mijn hele leven lang staan jullie voor me klaar, onvoorwaardelijk en altijd. Opvoeden is, net als geduld, een schone zaak. Jullie zijn erin geslaagd om van je gezin iets moois te maken. Jan en ik zijn allebei goed terecht gekomen, dat is jullie verdienste. Dank jullie wel voor alles wat jullie tot op de dag van vandaag voor me gedaan hebben en nog doen. Dank voor de support.
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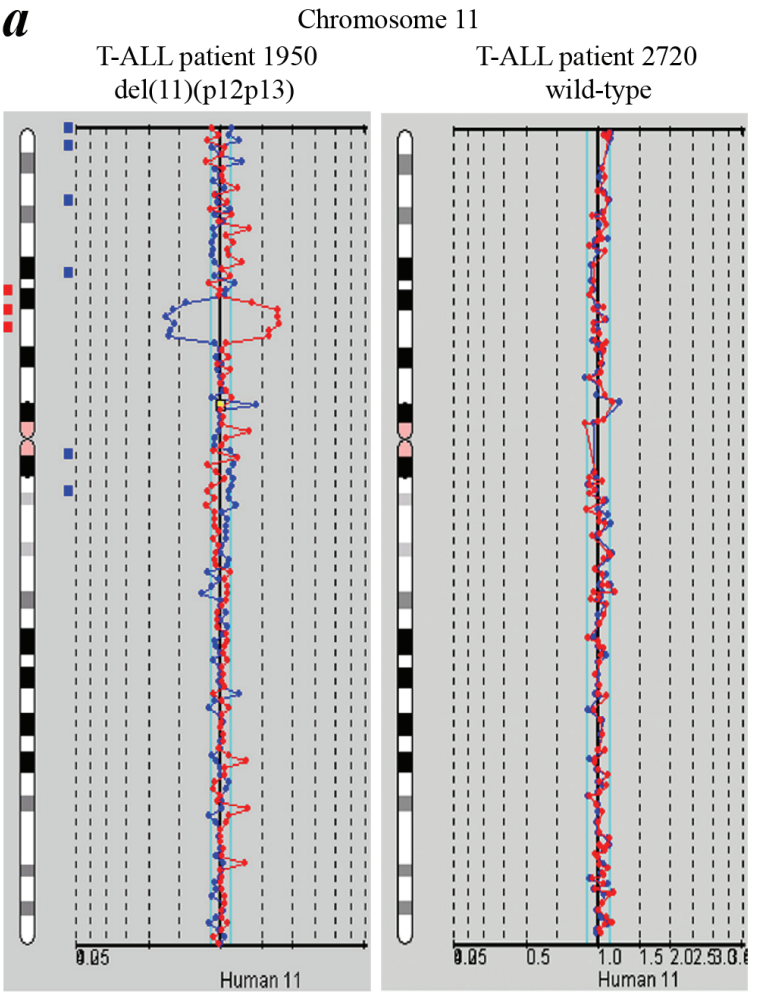
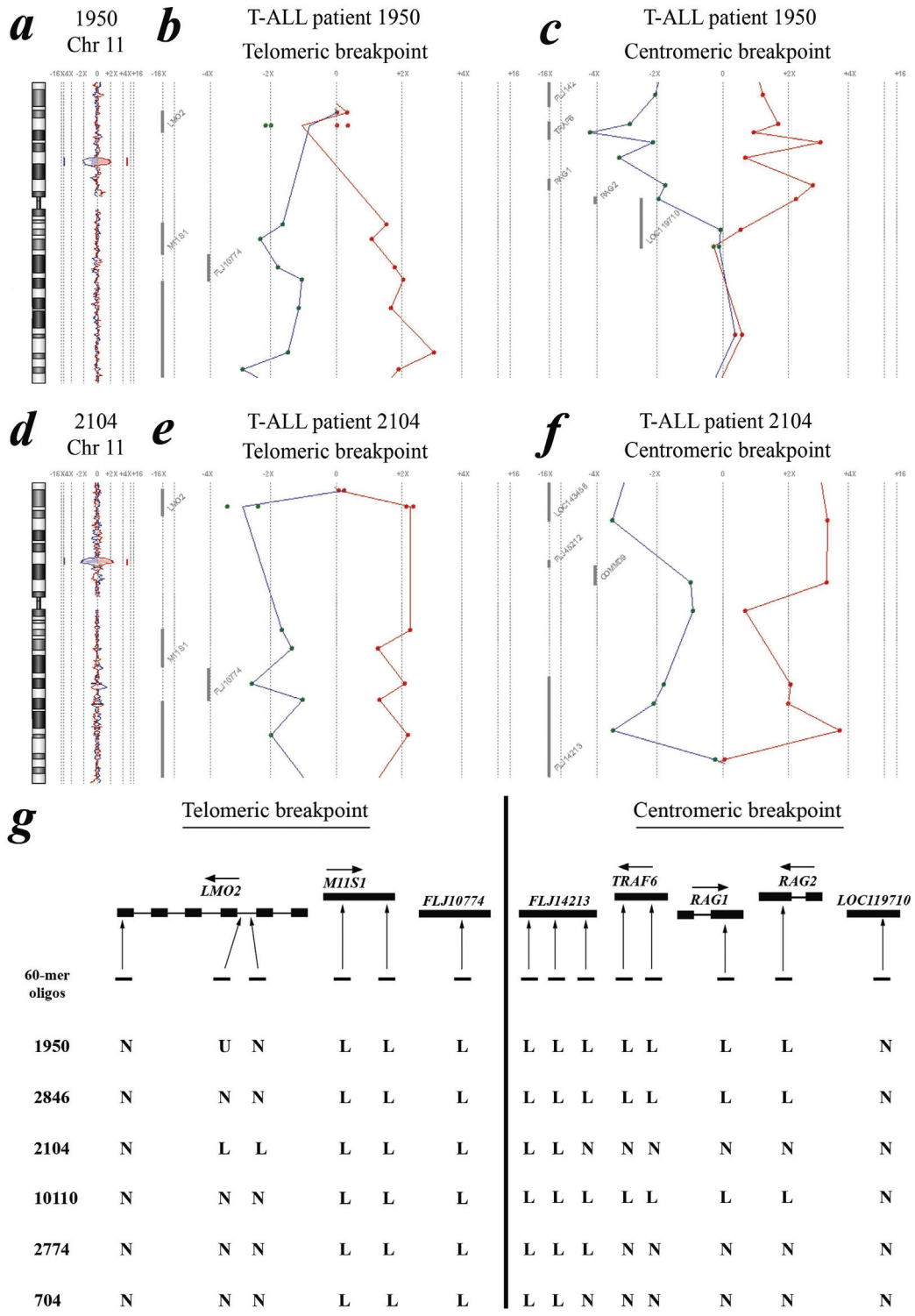
F

Colour figures



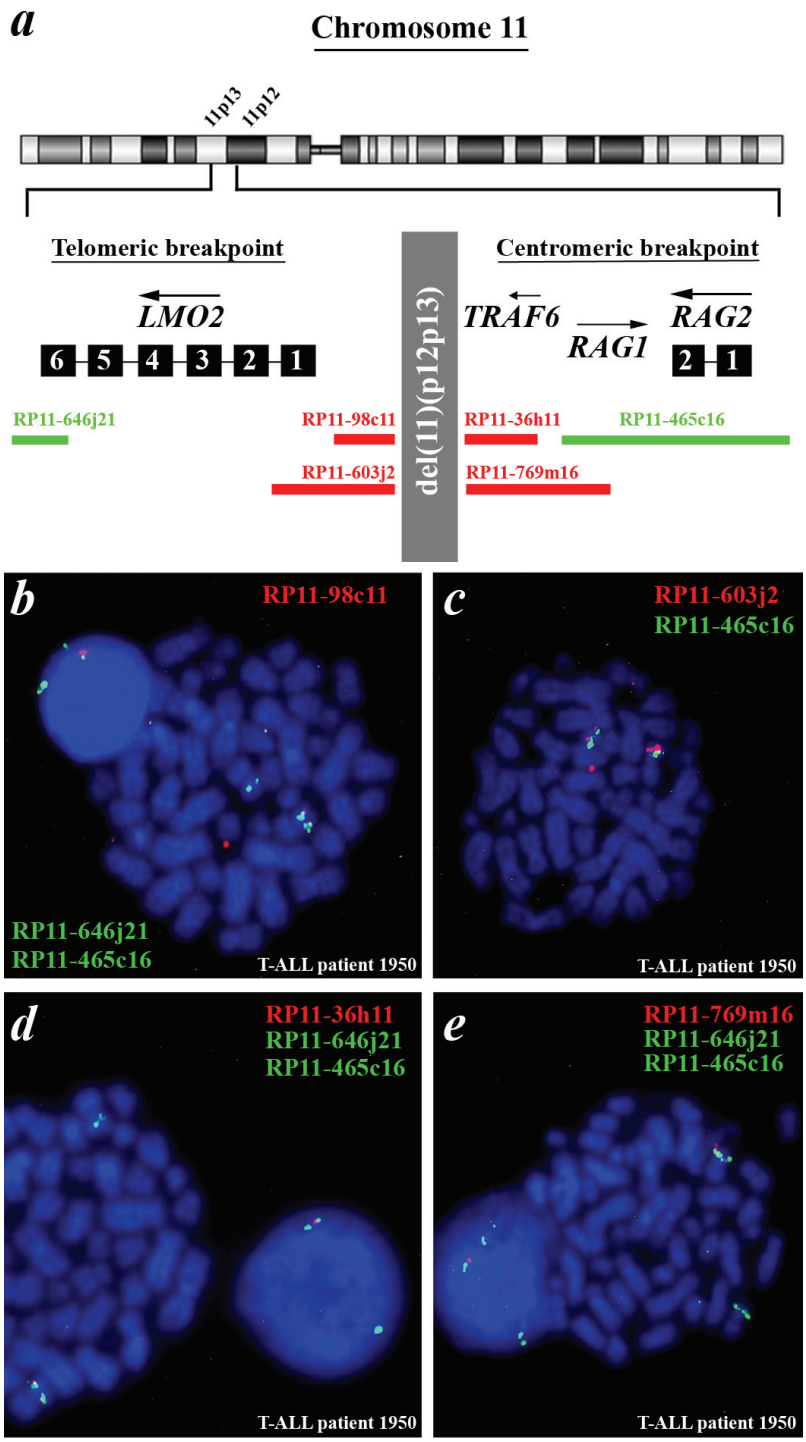
1950	Normal	Loss	Loss	Loss	Loss	Loss	Normal
2846	Normal	Loss	Loss	Loss	Loss	Loss	Normal
2104	Normal	Loss	Loss	Loss	Loss	Loss	Normal
10110	Normal	Loss	Loss	Loss	Loss	Loss	Normal
2774	Normal	Normal	Loss	Loss	Loss	Loss	Normal
704	Normal	Normal	Loss	Loss	Loss	Loss	Normal

Chapter 2, Figure 1. New recurrent deletion, del(11)(p12p13), in pediatric T-ALL (see page 35)
(A) Chromosome 11 ideogram and corresponding BAC array-CGH plot of test DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for T-ALL patients 1950 (left panel) and 2720 (right panel). (B) Overview of BAC array–CGH results for the 11p12-11p13 region for the 4 DCOG and the 2 COALLT-ALL patients with del(11)(p12p13). The BAC clones present on the DNA array and located on chromosome bands 11p12-11p13 are shown. Specific genes located in this region are indicated. Depicted genome positions are based on the UCSC Genome Browser at http://genome.ucsc.edu/.



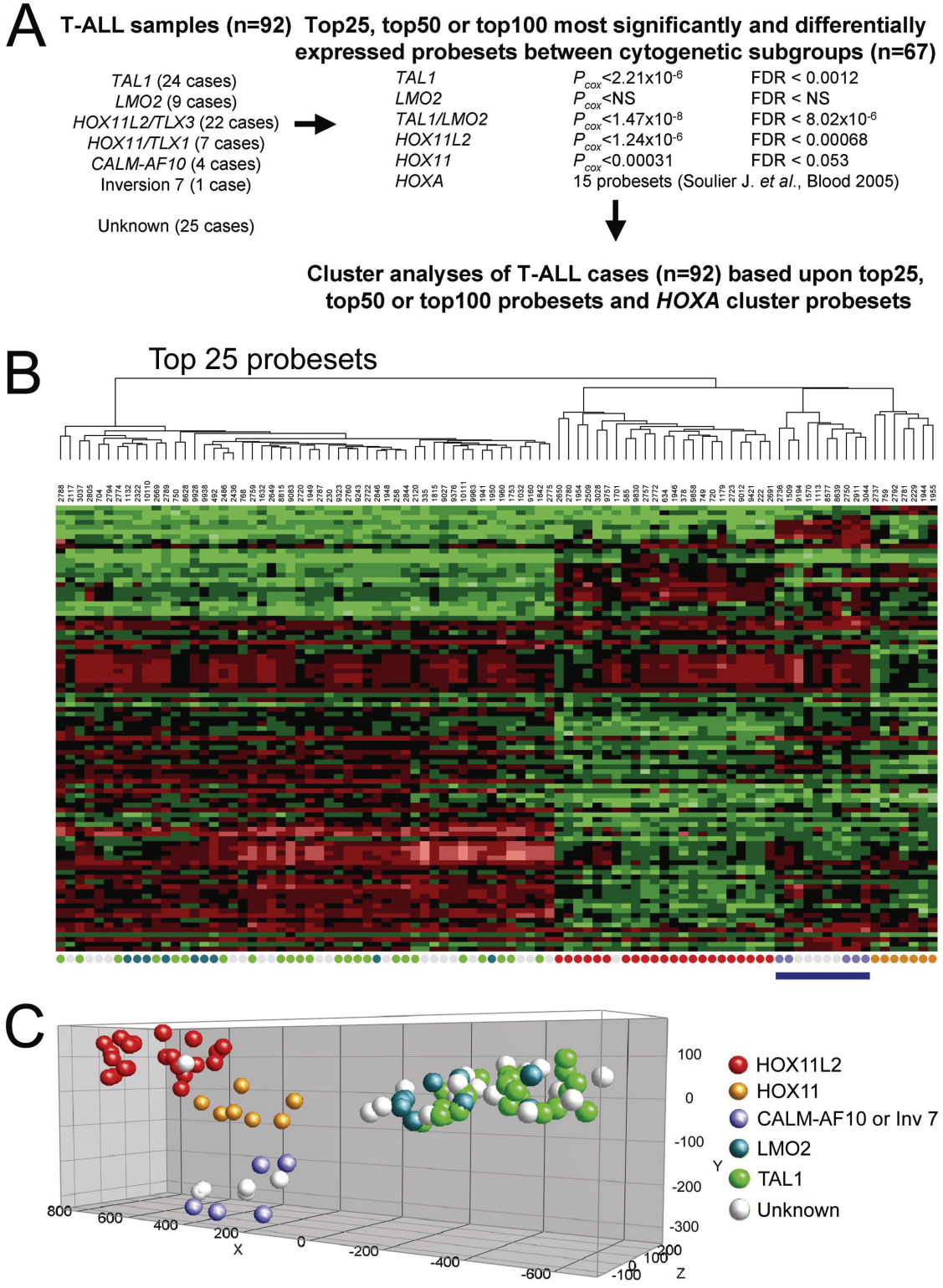
Chapter 2 Figure 2. Molecular characterization of deletion, del(11)(p12p13), in 6 pediatric T-ALL patients (see page 36)

Chromosome 11 ideogram and corresponding oligo array–CGH plot of test DNA/control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for T-ALL patient 1950 (A) and patient 2104 (D). Hybridization signals in the absence of amplifications or deletions scatter around the "zero" line, indicating equal hybridization for patient and reference DNA. Hybridization signals around the -2X or +2X lines represent loss of the corresponding region in the patient DNA. Detailed analysis of the telomeric breakpoints in patients 1950(B) and 2104(E) and the centromeric breakpoints in patients 1950 (C) and 2104 (F) of the deletion, del(11)(p12p13). (G) Overview of oligo array-CGH results in the potential breakpoint regions for 4 DCOG and the 2 COALL T-ALL patients with del(11)(p12p13). The 60-mer oligos present on the DNA array and located in the telomeric and centromeric breakpoint regions, as well as the specific genes located in this region with their transcription direction, are shown. N indicates normal; L, loss; and U, noninformative.

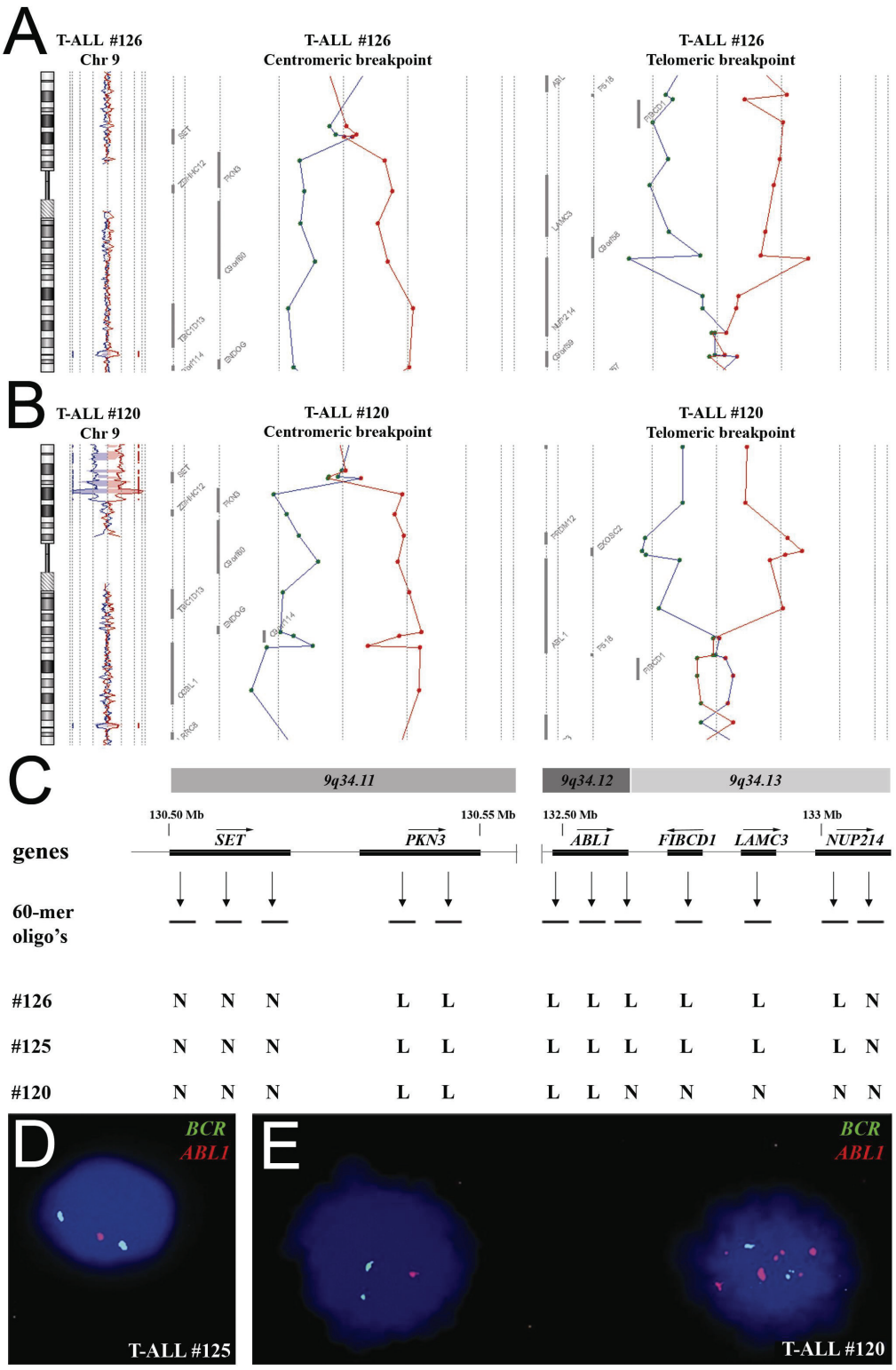


Chapter 2, Figure 3. FISH analysis confirms the presence of del(11)(p12p13) in T-ALL patient 1950 (see page 38)

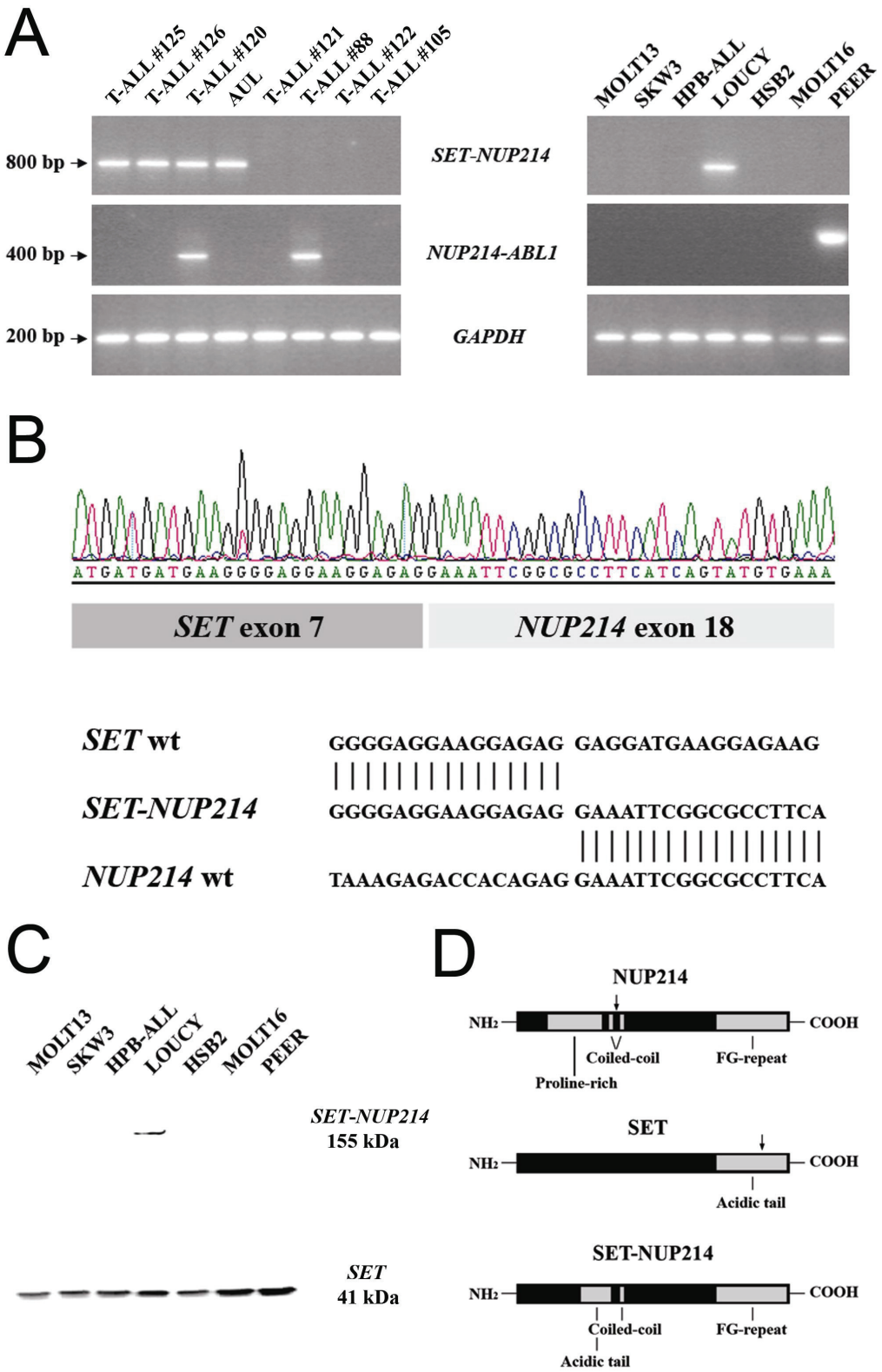
(A) Chromosome ideogram and overview of the genomic position of the BAC clones used for FISH analysis, located in the telomeric and centromeric breakpoint regions. (B) Dual-color FISH analysis on metaphase spreads of patient 1950 using RP11-465C16 (green), RP11-646J21 (green), and RP11-98C11 (red). The wild-type allele of chromosome 11 shows 2 green and 1 red signal, whereas on the mutated allele the red signal is lost and both green signals fuse. The extrachromosomal red signal represents background. (C) Dual-color FISH analysis on metaphase spreads of the same patient using RP11-465C16 (green) and RP11-603J2 (red). The intensity of the red signal is lower compared with the wild-type allele of chromosome 11, suggesting that only part of RP11-603J2 is deleted. (D) Dual-color FISH analysis on metaphase spreads using RP11-465C16 (green), RP11-646J21 (green), and RP11-36H11 (red). The wild-type allele of chromosome 11 shows 2 green and 1 red signal, whereas on the mutated allele the red signal is lost and both green signals fuse. (E) Dual-color FISH analysis on metaphase spreads using RP11-465C16 (green), RP11-646J21 (green), and RP11-769M16 (red). The wild-type allele of chromosome 11 shows 2 green and 1 red signal, whereas on the mutated allele the red signal is lost and both green signals fuse.



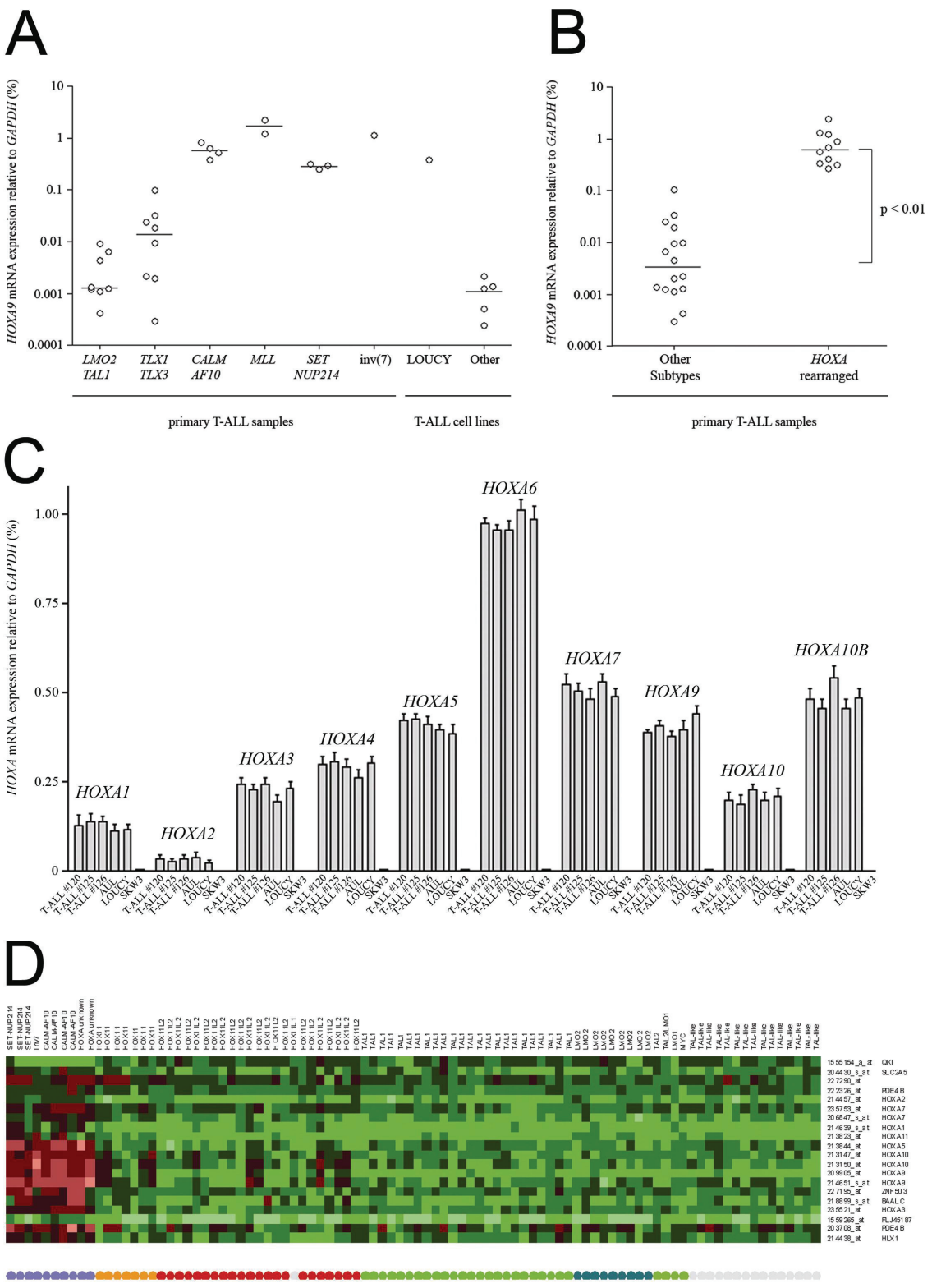
Chapter 3, Figure 1. Gene expression profiles of 92 T-ALL patients (see page 57)
(A) Differentially expressed genes among the major molecular-cytogenetic T-ALL subgroups (TAL1, LMO2, HOXA, HOX11/TLX1, and HOX11L2/TLX3; n=67). The significance level (Wilcoxon p value) and FDR corrected p value for the top 100 genes in each T-ALL subgroup is indicated. TAL1, HOX11, and HOX11L2 subgroups show significant differentially expressed probesets. (B) Cluster analysis of 92 patients with T-ALL (67 with known, 25 with unknown) based upon the top 25 most significant probesets for the TAL1, TAL1/LMO2, HOX11, and HOX11L2 subgroups combined with 15 HOXA probesets as previously described.[8] (C) Principal component analyses shows clustering of the patients with unknown T-ALL along the molecular-cytogenetic known patients: 1HOX11L2-like, 19 TAL1/LMO2-like, and 5 HOXA-like patients.



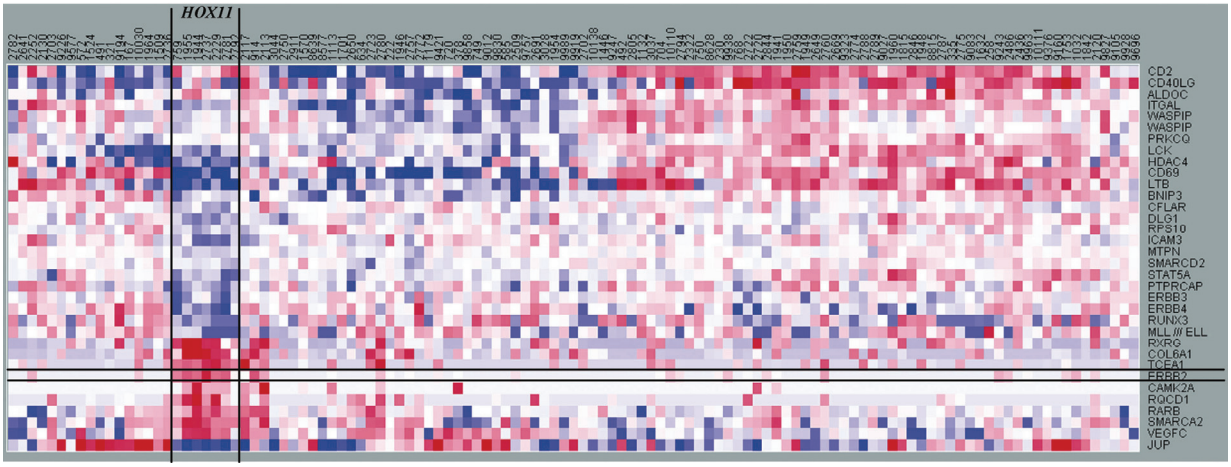
Chapter 3, Figure 2. Submicroscopic del(9)(q34.11q34.13) in T-ALL (see page 59)
(A) Chromosome 9 ideogram and corresponding oligo array-CGH plots of test DNA–control DNA ratios (blue tracing) versus the dye-swap experiment (red tracing) for patient no. 126. Detailed analyses of the centromeric and telomeric breakpoints show involvement of SET and NUP214. (B) Similar array-CGH plot for patient no. 120. Centromeric and telomeric breakpoints show involvement of SET and ABL1. (C) Overview of oligoarray-CGH results in the potential breakpoint regions for 3 patients with T-ALL with del(9)(q34.11q34.13). The 60-mer oligonucleotide probes present on the arrayCGH slide and located in the telomeric and centromeric breakpoint regions, as well as the specific genes located in this region with their transcription direction, are shown. Abbreviations: N; normal, L; loss. Dual-color FISH analysis of patient no. 125 (D) and no. 120 (E) using the LSI BCR-ABL ES translocation probe.



Chapter 3, Figure 3. ***SET-NUP214*** **fusion transcript in T-ALL** (see page 61)
RT-PCR analysis using SET- and NUP214-specific primers and GAPDH primers as internal control, reveals a specific SET-NUP214 fusion gene in T-ALL patient nos. 125, 126, and 120; the patient with AUL; and the T-ALL cell line LOUCY. NUP214-ABL1 fusion was detected in patient nos. 120 and no. 88 and in the T-ALL cell line PEER (B) Sequence analysis confirmed an identical fusion between exon 7 of SET and exon 18 of NUP214 in all SET-NUP214^{+} patients with T-ALL, the patient with AUL, and the LOUCY cell line. (C) Western blot analysis of T-ALL cell lines revealed a SET-NUP214 fusion in the cell line LOUCY. (D) At the protein level, the breakpoints are situated in the acidic tail of SET and the coiled-coil domain of NUP214.



Chapter 3, Figure 4. ***HOXA* activation in *SET-NUP214*$^{+}$ T-ALL** (see page 64)
(A) Relative HOXA9 expression levels by RQ-PCR for MLL-rearranged, CALM-AF10^{+}, inv(7)(p15q34), TAL1-, LMO2-, HOX11L2-, or HOX11-rearranged patients and T-ALL cell lines including LOUCY, MOLT13, SKW3, HPB-ALL, HSB2, and PEER. (B) Comparison of HOXA9 expression levels between the HOXA T-ALL subgroup (MLL, CALM-AF10, inv(7)(p15q34), and SET-NUP214; n=10) and other T-ALL subgroups (TAL1, LMO2, HOX11L2, or HOX11). The horizontal lines represent the medians. (C) Relative expression levels of HOXA genes by RQ-PCR for the 3 SET-NUP214^{+} patients with T-ALL, the LOUCY cell line, the patient with AUL, and SKW3. (D) Heatmap of the 20 significant and differentially expressed probesets with a false discovery rate (FDR) lower than 5% for the HOXA cluster compared with the other patients with T-ALL.



Chapter 6, Figure 1. Expression profile (heatmap) showing up-regulation of ERBB2 in a specific subgroup of pediatric T-ALL patients (see page 107)

Patients are shown in columns, genes are presented in rows. Red color indicates that a gene is upregulated, whereas blue indicates that this gene is downregulated. The HOX11-positive T-ALL patients as well as the ERBB2 gene are indicated between lines.