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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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.

Key words: pediatric T-ALL, *HOX11L2*, *CALM-AF10*, *TAL1*, outcome.

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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 ALL7/8 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's 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, glyceraldehyde-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-

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*	*Remaining***
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, $10\times^{9}$/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.*

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 ≤50×10^{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

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

Pat. nr	M/F	Age (yrs)	WBC $\times 10^9$/L	Protocol	SIL-TAL RT-PCR	SIL-TAL FISH	HOX11L2 RT-PCR	HOX11L2 FISH	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	Del	0	wt	0	wt	0	0	wt	wt	46,XY[32]	CCR	44	alive
2	F	8	132	ALL9	0.014	Del	0	wt	0	wt	0	0	wt	wt	46,XX,?add(9)(p1?)[6]/46,XX[30]	CCR	35	alive
3	M	12	110	ALL7	0.022	Del	0	wt	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	15	300	ALL8	0,062	ND	0	ND	0	ND	0	0	wt	ND	46,XY[19]	CCR	139	alive
5	M	4.3	205	ALL8	0.022	Del	0	ND	0	ND	0	0	wt	ND	45,XY,del(6)(q15q25),dic(9;13)(p11;q13)[34]	CCR	105	alive
6	M	15	200	ALL8	0	Trans	0	wt	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	192	ALL8	0	Trans	0	wt	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	Del	0	wt	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	Del	0	wt	0	wt	0	0	wt	wt	46,XY[30]	CCR	56	alive
10	M	10	112.2	ALL9	0.045	Del	0	wt	0	wt	0	0	wt	wt	46,XY,dup(17)(q12q24)[14]	CCR	41	alive
11	M	13	41,4	ALL9	0.090	Del	0	wt	0	wt	0	0	wt	wt	45,XY,-14[13]/46,XY[16]	CCR	31	alive
12	M	9.3	310	ALL9	0	Trans	0	wt	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	Del	0	wt	0	wt	0	0	wt	wt	46,XX[28]	CCR	2	td
14	M	6.4	188.1	ALL9	0.008	Del	0	wt	0	wt	0	0	wt	wt	46,XY[30]	CCR	54	alive
15	M	5.5	185	ALL9	0	wt	0.27	Trans	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	wt	0.40	Trans	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	wt	1.50	Trans	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	wt	1.84	Trans	0	wt	0	0	wt	wt	46,XY	R	12	dod
19	F	7.8	89,5	ALL8	0	ND	1.19	Trans*	0	ND	0	0	wt	ND	46,XX[20]	CCR	122	alive
20	M	5.8	500	ALL7	0	ND	1.39	Trans	0	ND	0	0	wt	ND	UNKN	CCR	50	alive
21	M	5	140	ALL8	0	wt	1.43	Trans	0	wt	0	0	wt	wt	46,XY(100%)	R	12	dod
22	M	7.2	354	ALL8	0	ND	17.56	Trans*	0	ND	0	0	wt	ND	46,XY[6]	CCR	61	alive
23	M	6.3	35.9	ALL9	0	wt	0.87	Trans*	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	wt	8.22	Trans*	0	wt	0	0	wt	wt	46,XY[10]	R	11	dod
25	F	6.4	98	ALL8	0	wt	0.39	wt	0	wt	0	0	wt	wt	45,XX,dic(7;8)(p12;p12)[7]	R	8	dod
26	F	12	34.1	ALL9	0	wt	0.28	Trans	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	wt	1.23	Trans	0	wt	0	0	wt	wt	46,XY,t(1;7)(p34;q34)[15]	R	9	dod
28	M	5.1	80	ALL9	0	wt	0.51	Trans	0	wt	0	0	wt	wt	46,XY[30]	CCR	40	alive
29	M	5.3	31.8	ALL9	0	wt	0.27	Trans	0	wt	0	0	wt	wt	46,XY[32]	CCR	41	alive
30	F	8.8	88.5	ALL8	0	wt	0.27	Trans	0	wt	0	0	wt	wt	46,XX(100%)	CCR	75	alive
31	M	5.1	44.9	ALL8	0	wt	1.69	Trans	0	wt	0	0	wt	wt	47,XY,+8[4]	R	17	dod
32	M	10	149	ALL9	0	wt	0	wt	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	0	wt	0.048	Trans	0	0	wt	wt	46,XX	CCR	9	td
34	M	7.8	159	ALL8	0	wt	0	wt	0.189	Trans	0	0	wt	wt	46,XY,t(7;10)(q36;q25-q26)	CCR	80	alive
35	M	11	53,9	ALL9	0	wt	0	wt	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	0	wt	0.334	Trans	0	0	wt	wt	46,XY[32]	CCR	95	alive
37	M	3.5	56,6	ALL9	0	wt	0	wt	0.237	Trans	0	0	wt	wt	47,XY,+del(9)(p1?1)[11]	CCR	62	alive
38	M	11	347	ALL9	0	wt	0	Trans*	0	wt	0.013	0	Trans	wt	46,XY,t(10;11)(p13;q21)[20]	R	22	dod
39	M	10	13,7	ALL9	0	wt	0	wt	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	0	wt	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	0	wt	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	0	wt	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	0	wt	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	NE	0	wt	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	17	86.3	ALL9	0	wt	0	wt	0	wt	0	0	wt	wt	46,XX,del(6)(q13q21),t(8;14)(q24;q11),del(9)(p22)	CCR	22	td
46	M	16	128.7	ALL9	0	wt	0	wt	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	16	136	ALL9	0	wt	0	wt	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	0	wt	0	wt	0	0	wt	wt	46,XX,del(6)(q?)[8]	CCR	119	alive
49	M	14	600	ALL9	0	wt	0	wt	0	wt	0	0	wt	wt	46,XY[26]	R	14	dod
50	F	8.4	15.4	ALL9	0	wt	0	wt	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	0	wt	0	wt	0	0	wt	wt	46,XX[28]	CCR	31	alive
52	M	14	UNKN	ALL8	0	ND	0	ND	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	0	ND	0	ND	0	0	wt	ND	47,XY,+8	CCR	116	alive
54	F	4.3	5.3	ALL8	0	ND	0	ND	0	ND	0	0	wt	ND	46,XX,del(14)(q21q31)[4]	CCR	98	alive
55	M	6.2	167.2	ALL8	0	wt	0	wt	0	wt	0	0	wt	wt	47,XY,+?6[6]	R	7	dod
56	M	2.8	649	ALL7	0	ND	0	ND	0	ND	0	0	wt	ND	UNKN	CCR	18	alive
57	M	3.8	91.5	ALL8	0	NE	0	wt	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)	CCR	89	alive
58	F	1.9	387	ALL8	0	ND	0	ND	0	ND	0	0	wt	ND	46,XX[30]	CCR	96	alive
59	M	9.1	226	ALL8	0	wt	0	wt	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	0	wt	0	wt	0	0	wt	wt	UNKN	CCR	87	alive
61	M	6.2	191	ALL9	0	wt	0	wt	0	wt	0	0	wt	wt	46,XY[30]	CCR	39	alive
62	M	3.9	435	ALL9	0	wt	0	wt	0	wt	0	0	wt	wt	46,XY[30]	CCR	18	td
63	F	4.7	212	ALL8	0	wt	0	wt	0	wt	0	0	wt	wt	46,XX[32]	R	9	dod
64	F	16	231	ALL9	0	wt	0	wt	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	49.5	ALL8	0	wt	0	wt	0	wt	0	0	wt	wt	46,XX	CCR	73	alive
66	M	9	95.2	ALL9	0	wt	0	wt	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	0	wt	0	wt	0	0	wt	wt	46,XY[31]	CCR	44	alive
68	M	1.8	250	ALL9	0	wt	0	wt	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	0	wt	0	wt	0	0	wt	wt	46,XY[20]	CCR	51	alive
70	M	14	128.9	ALL9	0	wt	0	wt	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	0	wt	0	wt	0	0	wt	wt	46,XY[31]	R	8	dod
72	F	5.3	60	ALL9	0	wt	0	wt	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.*

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.

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-AF6*), 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.

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 fre-

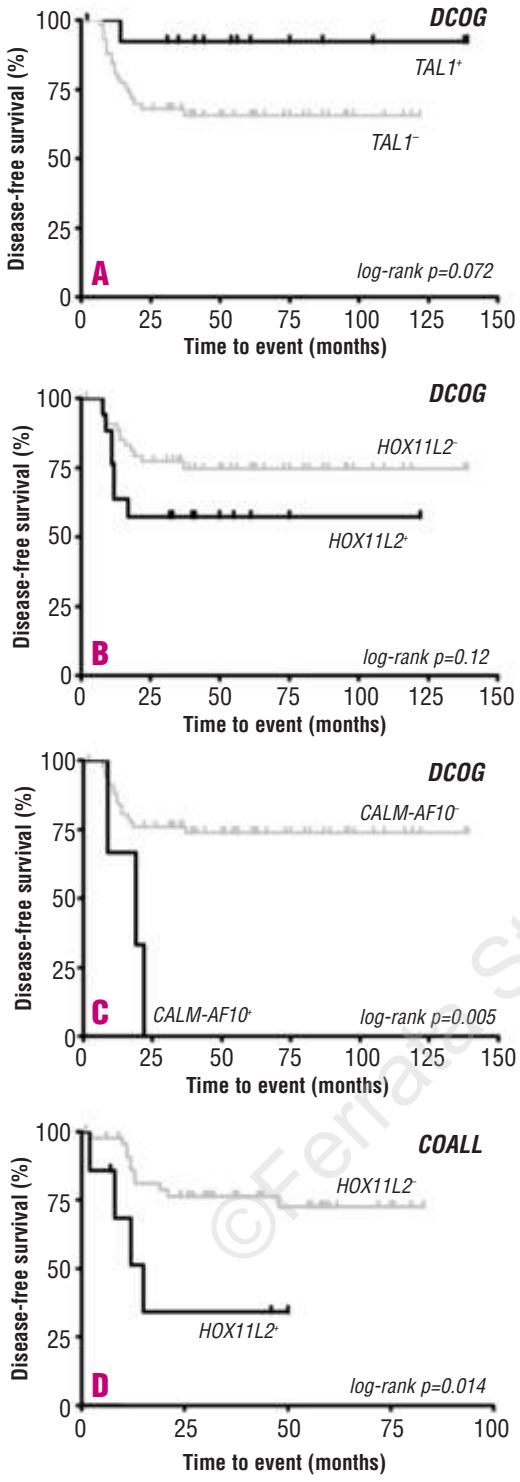

Figure 1. Disease-free survival of pediatric T-ALL patients from the DCOG cohort (n=72). Disease-free survival for (A) the *TAL1* rearranged (*TAL1*$^+$) versus non-*TAL1* rearranged (*TAL1*$^-$), for (B) *HOX11L2* rearranged (*HOX11L2*$^+$) versus non-*HOX11L2* rearranged (*HOX11L2*$^-$); (C) *CALM-AF10*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

quently 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), TCRαβ 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 TCRαβ 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 TCRαβ. 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

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.

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*).

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*

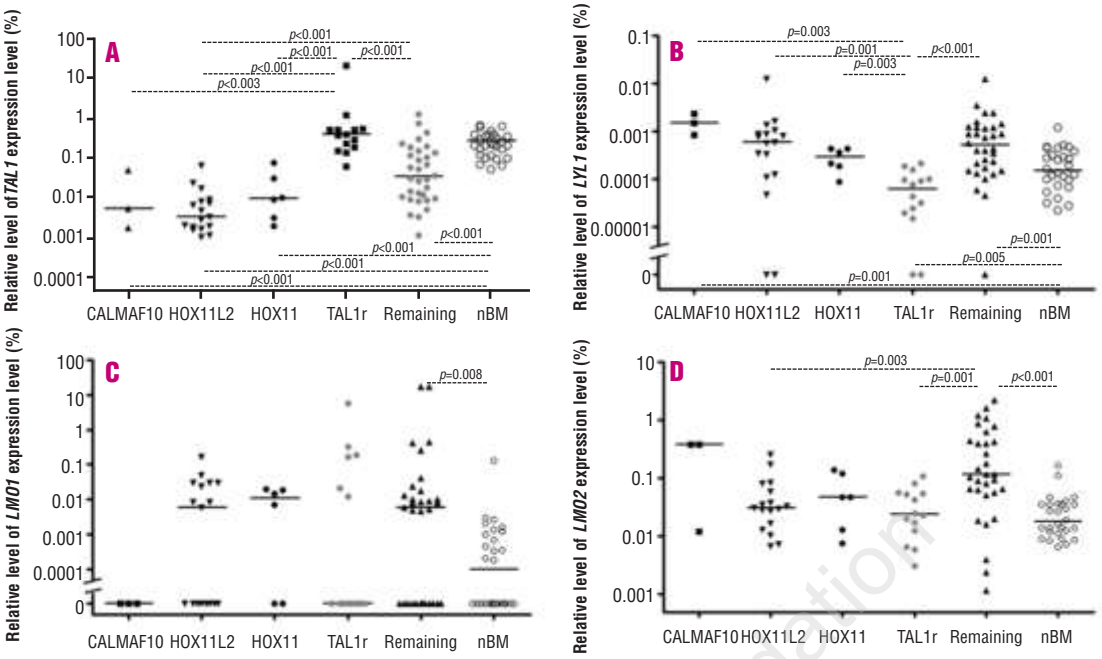

Figure 2. Expression levels of *TAL1*, *LYL1* and *LMO2* in T-ALL subgroups. Relative levels of expression of (A) *TAL1*, (B) *LYL1*, (C) *LMO1* and (D) *LMO2* as percentage of expression of the housekeeping gene *GAPDH* for the molecular-cytogenetically distinct T-ALL subgroups (*CALM-AF10, HOX11L2,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

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*-subgroups 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 TCRαβ positive late cortical development stage[6,7,11] which, in turn, corresponds to the αβ-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-αβ 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 *NUP214-ABL1* amplifications.[45] One of the *HOX11L2*-rearranged patients in our DCOG cohort (patient #20) had a *NUP214-ABL1* 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 *ABL1* abnormalities. In a pilot screening, we also investigated whether the presence of activating mutations in *NOTCH1*, 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.

MVG: wrote manuscript, designed research, performed experiments; JPPM: wrote manuscript, requested grant, designed research; HBB: designed research, critical discussion; AWL: designed research, critical discussion; JGCAMB-G: technical assistance; PS: technical assistance; TSP: designed research, technical assistance; MLdB: data handling COALL study group, critical discussion; MH: study leader COALL, critical discussion; WAK: study leader DCOG ALL7/ALL8, critical discussion; AJPV: study leader DCOG ALL9, critical discussion; ERvW: provided samples and clinical data, critical discussion; MMvN: wrote manuscript, designed research; RP: wrote manuscript, requested grant, designed research.

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