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A new recurrent inversion, inv(7)(p15q34), leads to transcriptional activation of *HOXA10* and *HOXA11* in a subset of T-cell acute lymphoblastic leukemias

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Chromosomal translocations with breakpoints in T-cell receptor (*TCR*) genes are recurrent in T-cell malignancies. These translocations involve the *TCRαδ* gene (14q11), the *TCRβ* gene (7q34) and to a lesser extent the *TCRγ* gene at chromosomal band 7p14 and juxtapose T-cell oncogenes next to *TCR* regulatory sequences leading to deregulated expression of those oncogenes. Here, we describe a new recurrent chromosomal inversion of chromosome 7, inv(7)(p15q34), in a subset of patients with T-cell acute lymphoblastic leukemia characterized by CD2 negative and CD4 positive, CD8 negative blasts. This rearrangement juxtaposes the distal part of the *HOXA* gene cluster on 7p15 to the *TCRβ* locus on 7q34. Real time quantitative PCR analysis for all *HOXA* genes revealed high levels of *HOXA10* and *HOXA11* expression in all inv(7) positive cases. This is the first report of a recurrent chromosome rearrangement targeting the *HOXA* gene cluster in T-cell malignancies resulting in deregulated *HOXA* gene expression (particularly *HOXA10* and *HOXA11*) and is in keeping with a previous report suggesting *HOXA* deregulation in *MLL*-rearranged T- and B cell lymphoblastic leukemia as the key factor in leukaemic transformation. Finally, our observation also supports the previous suggested role of *HOXA10* and *HOXA11* in normal thymocyte development.
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Introduction

T-cell acute lymphoblastic leukemia (T-ALL) represents 10–15% of childhood and 25% of adult ALLs, and are associated with an intermediate prognosis within the total group of ALLs.[1] In T-ALL, chromosomal rearrangements affecting the T-cell receptor loci *(TCR)* were among the first to be detected. Similar to translocations involving the *IGH* locus, these chromosomal changes cause ectopic expression of target genes due to juxtaposition to *TCR* gene enhancers. These pivotal studies showed that T-ALLs were genetically very heterogeneous with the most frequently detected translocation, the t(11;14)(p15;q11), being found in less than 10% of cases.[2] One particular rare translocation, t(1;14)(p32;q11), lead to the discovery of the *TAL1(SCL)* gene that turned out to be of crucial importance in normal T-cell development. Most interestingly, *TAL1(SCL)* was later shown to be transcriptionally activated due to a cryptic 90 kb interstitial deletion in as much as 20% of T-ALLs, thus representing the most frequent genetic abnormality in this disease.[3] Similarly, other T-cell leukemia specific genes (*HOX11, LYL1, LMO1, LMO2, HOX11L2*), initially identified through rare chromosomal rearrangements involving *TCR* loci, were also shown to be transcriptionally activated in T-ALLs without evidence for particular translocations. Also, recent studies showed biallelic overexpression of some of these genes thus further supporting the notion for a broader implication in T-ALL through mechanisms other than the known translocation events.[4] Furthermore, the unexpected finding of amplification of a *NUP214/ABL1* fusion gene in a minority of T-ALL patients,[5,6] further illustrated the diversity of genetic events involved in T-ALLs. Likewise, the *NOTCH1* gene, involved in the t(7;9)(q34;q34) in less than 1% of T-ALLs, was shown to harbor activating mutations in more than 50% of T-ALLs.[7]

Although involvement of each of the three TCR loci in recurrent chromosomal changes in T-ALL is well recognized,[8] both the incidence and the exact contribution of each of these loci in recurrent chromosome abnormalities have not yet been assessed in detail. The finding of a previously unreported t(7;11)(q34;q24) in a child with T-ALL, as the result of a multicolor fluorescence *in situ* hybridization (M-FISH) study on T-ALLs, prompted us to screen a large cohort of 94 patients with T-ALL. This resulted in the finding of a new recurrent chromosomal rearrangement, that is, a pericentric inversion of chromosome 7, inv(7)(p15q34), in a subset of T-ALL patients. This inversion was associated with an elevated expression of *HOXA10* and *HOXA11*, most probably due to juxtaposition near strong enhancers embedded within the *TCRβ* locus. This is the second report providing evidence for involvement of class I *HOXA* genes in the development of T-ALL.

Materials and methods

Patients and thymocytes

Diagnostic bone marrow, peripheral blood or pleural fluid samples from 49 adults and 45 children with T-ALL were collected from three institutes, retrospectively. For 32 T-ALL cases (16 children and 16 adults) frozen cells were available for expression analysis. These selected cases contained all cases positive for *TCRβ* chromosomal rearrangement and 13 cases

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Table 1 Biological characteristics and cytogenetic findings of TCRβ positive (case n° 1–19; with identified partner gene) and negative T-ALL patients (case n° 20–32)

Case no.	*Age*	*Sex*	*Karyotype*	*TCRβ partner gene*	*Revised karyotype*
1	4	M	46,XY, t(1;14)(p32;q11) [20]	Unknown	46,XY, t(1;14)(p32;q11), t(6;10)(q25;q24), t(7;11)(q34;q24)[a]
2	19	M	46, XY, t(1;7)(p32;q34), del(6)(q12q16) [14]/46, XY [1]	*TAL1* (1p32)	46, XY, t(1;7)(p32;q34), del(6)(q12q16) [14]/46, XY [1]
3	45	M	46,XY [9]	Unknown	46,XY, t(7;?)(q34;?) [9]
4	6	M	47,XY,+8,del(9)(p21) [3]/46,XY [8]	Unknown	47,XY, t(7;?)(q34;?),+8, del(9)(p21) [3] /46,XY [8]
5	12	F	46, XX,del(6)(q23q26),add(7)(q31),del(11)(q13), −14,+mar mar = ?der(1) i(1)(q10)add(1)(q32) [18]/92, idem x 2 [2]	Unknown	46, XX,del(6)(q23q26), t(7;?)(q34;?),del(11)(q13),−14,+mar mar = ?der(1) i(1)(q10)add(1)(q32) [18]/92, idem x 2 [2]
6	6	M	46,XY,del(7)(q21),?inv(14)(q11q32) [14]/46,XY [6]	Unknown	46,XY,t(7;?)(q34;?),?inv(14)(q11q32) [14]/46,XY [6]
7	15	F	46,XX [20]	*RBTN2* (11p13)	46,XX, t(7;11)(q34;p13) [20]
8	10	M	46,XY [20]	Unknown	46,XY, t(7;?)(q34;?) [20]
9	9	F	46,XX,del(7)(q34),t(10;14)(q22;q32) [11]	*RBTN1* (11p15)	46,XX,t(7;11)(q34;p15),t(10;14)(q22;q32) [11]
10	9	M	46,XY,t(8;14)(q24;q11) [8]/46,XY [9]	*RBTN1* (11p15)	46,XY,t(8;14)(q24;q11), t(7;11)(q34;p15) [8]/46,XY [9]
11	26	M	46,XY[20]	Unknown	46,XY, t(7;?)(q34;?) [20]
12	15	M	47,XY,t(7;9)(q34;q34),+20 [2]/47, idem, add(11)(q24) [9]	*TAN1* (9q34)	47,XY,t(7;9)(q34;q34),+20 [2]/47, idem, add(11)(q24) [9]
13	30	M	46–47,XY,+mar1, +mar2, [cp5]	Unknown	46–47,XY, t(7;?)(q34;?), +mar1, [cp5]
14	23	M	42–48,XY,t(3;11)(p12;p15),t(7;10)(q35;q24), t(8;10)(q21;q?24), +11, +12 [5]/46,XY [4]	*HOX11* (10q24)	42–48,XY,t(3;11)(p12;p15),t(7;10)(q35;q24), t(8;10)(q21;q?24), +11, +12 [5]/46,XY [4]
15	35	F	46,XX [4] failure	*HOXA* (7p15)	46,XX, inv(7)(p15q34) [4]
16	27	F	46, XX, add(5)(q31) [11]/ 46, XX [9]	*HOXA* (7p15)	46, XX, add(5)(q31), inv(7)(p15q34) [11]/ 46, XX [9]
17	15	M	46,XY [20]	*HOXA* (7p15)	46,XY, inv(7)(p15q34) [20]
18	34	M	46,XY [20]	*HOXA* (7p15)	46,XY, inv(7)(p15q34) [20]
19	49	M	47,XY,del(6)(q14),del(7)(p?),del(9)(p21),+mar1 [4]/46,XY [6]	*HOXA* (7p15)	47,XY,del(6)(q14),inv(7)(p15q34),del(9)(p21), +mar1 [4]/46,XY [6]
20	35	M	46, XY, t(9;20)(p21;q12), t(10;14)(q24;q11), del(12)(p12) [13]/46, XY [12]	Negative	
21	26	M	46,XY,t(6;11)(q21–22;p15),add(12)(p13) [17]/46,XY [3]	Negative	
22	35	M	46, XY [20]	Negative	
23	27	M	47, XY, t(7;14)(p12;q24), del(9)(q21q31), +10 [16]/46, XY [4]	Negative	
24	35	M	46, XY [25]	Negative	
25	24	M	46, XY [25]	Negative	
26	45	M	47, XY, +8 [2]/ 46, XY[20]	Negative	
27	5	M	47,XY,del(6)(q12),+8, del(10)(q24)[5]/ 46,XY [18]	Negative	47,XY,del(6)(q12),+8, del(10)(q24), t(5;14)(q35;q32)* [5]/ 46,XY [18]
28	9	M	46,XY [20]	Negative	
29	9	M	46,XY [20]	Negative	
30	3	M	46,XY,add(19)(p13.3),del(6)(q15q24) [6]/ 46,XY [13]	Negative	
31	15	M	46,XY,add(14)(q32) [9]/46,XY [18]	Negative	
32	16	F	46,XX,del(1)(q32),del(5)(q22q34),−7,+mar [5]/46,XX [7]	Negative	

[a]Including M-FISH analysis.

without *TCRβ* rearrangement, which were investigated by FISH (see below). Table 1 lists biological and cytogenetic findings for these patients. Total child thymocyte suspension (CT) and purified CD34$^+$ thymus cells (CD34 + T) were isolated as described before.[9]

Immunophenotyping

Immunophenotypic studies were carried out in the respective centers according to established protocols. Cases were classified using the European Group for the Immunological Characterization of Leukemias (EGIL) recommendations.[10] Briefly, EGIL T1 (pro-T ALL) was defined by the presence of only CD7, T2 (pre-T ALL) by CD2 and/or CD5 and/or CD8 positivity, T3 (cortical T-ALL) by CD1a positivity and T4 (mature T-ALL) by the presence of surface CD3 and lack of CD1a.

Cytogenetic studies

The different diagnostic specimens (bone marrow, blood and pleural fluid) were cultured and harvested for cytogenetic analysis according to established methods. Chromosome slides were G-, Q- or R-banded. Chromosome aberrations are described according to guidelines of an International System for Human Cytogenetic Nomenclature (ISCN 1995).[11]

Fluorescence in situ *hybridization (FISH)*

Cytogenetic cell suspensions (methanol/acetic acid fixed cells) or unstained slides were available for all 94 patients. For FISH the following *TCRβ* (7q34) and *HOXA* (7p15) flanking BAC clones were selected: RP11-1220K2 (located centromeric to *TCRβ*) and RP11-556I13 (located telomeric to *TCRβ*); RP1-167F23 (containing telomeric *HOXA* genes *HOXA1*, *HOXA2* and part of *HOXA3*) and RP5-1103I5 (located centromeric to *HOXA*); RP11-1036C18, RP11-163M21, RP11-1132K14 and RP11-1025G19 clones represent a contig spanning the entire *HOXA* gene cluster (Figure 2). For further delineation of the *TCRβ* breakpoint, following gene covering clones were selected: RP11-785K24 (centromeric) and RP11-701D14 (telomeric) (Figure 3).

RPCI-11 (Human BAC Library) clones were provided by the Welcome Trust Sanger Institute (Cambridge, UK) and Invitrogen (Paisley, Scotland). Clone DNA isolation, labeling and FISH were performed as previously described,[12] using biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics Belgium, Vilvoorde, Belgium) as haptens.

Disruption of the *TCRβ* locus was assessed by dual color FISH with *TCRβ* flanking probes (RP11-1220K2 and RP11-556I13). Cases carrying *TCRβ* chromosomal rearrangement (identified by a split signal of the flanking probes) were further analyzed to confirm the presumed *TCRβ* partner genes using flanking probes for the respective partner loci (*RBTN1* on 11p15, *RBTN2* on 11p13, *HOX11* on 10q24, *TAN1* on 9q34, *TAL1(SCL)* on 1p32). For those cases, which showed inversion of the distal *TCRβ* flanking probe to 7p, dual color FISH using *HOXA* flanking probes was performed.

Hybridization signals were evaluated by two independent observers and interpreted at the interphase (100 nuclei) and metaphase level (when available).

RNA isolation, cDNA synthesis and quantitative real-time RT-PCR

Patients were selected retrospectively on the basis of the availability of frozen material at diagnosis. All human samples were obtained according to the guidelines of the local ethical committees. These included 13 *TCRβ* positive cases including five inv(7) positive and 13 *TCRβ* negative cases. Cells obtained from total child thymus (CT), and purified $CD34^+$ thymocytes ($CD34+$ T) were used as a reference for *HOX* gene expression in normal developing T-cells.[13] RNA was isolated using Trizol (Invitrogen, Merelbeke, Belgium) and RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase treatment, cDNA synthesis, primer design and SYBR Green I quantitative real-time RT-PCR were performed as previously described.[14] Reactions were performed on an ABI Prism 5700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR data analysis and expression normalization were performed using three internal control genes (*RPL13A, UBC, YWHAZ*), as described[15] and correlated to the mean expression level of each gene. Primers for *HOXA1, HOXA4, HOXA5, HOXA7, HOXA11* were designed according to Thompson *et al.*[16] Primers for the other *HOXA* genes (*HOXA2, HOXA3, HOXA6, HOXA9, HOXA10, HOXA13*) and for *HOXB3* and *HOXC4* expression analysis were designed and developed according to Wang *et al*[17] at the Primer Bank. Primer sequences for the tested genes are deposited in RTPrimerDB, a public database for real-time PCR primers (http://medgen.ugent.be/rtprimerdb) (RTPrimerDB).[18]

Data analysis

Statistical analysis was performed using SPSS Software (SPSS Inc., Chicago, IL, USA) version 12.0. The nonparametric Mann–Whitney *U* test (two-tailed) was used to evaluate the significance of difference in mean expression levels between the patients subgroups (inv(7) positive *vs* inv(7) negative patients). The Fisher exact test was used to evaluate the relationship between the specific immunophenotype and the presence of the inv(7).

Results

Frequency of chromosomal rearrangements affecting the TCRβ locus in T-ALL

A previously conducted M-FISH analysis in one T-ALL patient (case no. 1) with a known t(1;14)(p32;q11) and *TAL1(SCL)* overexpression, uncovered a cryptic translocation t(7;11)(q34;q24) affecting the *TCRβ* locus. No known T-cell oncogene is located on distal 11q and breakpoint analysis is currently ongoing. FISH screening for *TCRβ* chromosomal rearrangements in a large series of 93 T-ALLs yielded split signals for the *TCRβ* locus in 18 cases, which brings the total of detected *TCRβ* alterations to 20% (19/94). In six of these 19 cases, the distal probe for *TCRβ* was translocated to recurrent *TCRβ* partner genes: *TAL1* (case no. 2), *RBTN2* (case no. 7), *RBTN1* (cases no. 9–10), *TAN1* (case no. 12), *HOX11* (case no. 14) as confirmed by FISH with the appropriate probes (Table 1). In eight of the 19 cases showing *TCRβ* rearrangement, involvement of known partner genes was excluded. Interestingly, in five other cases with split signals for the *TCRβ* flanking probes, the telomeric BAC clone for *TCRβ* moved to the distal end of the short arm of chromosome 7, thus revealing the presence of a pericentric inversion with an unknown partner gene (Figure 1a).

Biological characteristics, immunophenotyping and cytogenetics

Biological characteristics for all *TCRβ* positive cases (cases no. 1–19) and selected *TCRβ* negative T-ALLs (case no. 20–32) are summarized in Table 1. *TCRβ* positive cases with inv(7), aged 15–49 years (median 32 years), showed an M/F ratio: 1.5:1.0. *TCRβ* positive cases without inv(7) showed a lower age of onset compared to inv(7) positive cases: median 16.6 years (range 4–45 years) and a pronounced male predominance (M/F ratio 3.6:1.0). Compared to inv(7) positive cases, *TCRβ* negative cases also showed a lower age of onset with a median of 21.8 years (range 3–45 years) and a striking male predominance (M/F ratio 12:1).

Immunophenotypic findings from all but two cases are listed in Table 2. Inv(7) positive cases were classified as either T3 ($n=1$) or T4 ($n=4$) T-ALL immunophenotypes but with a distinct pattern of CD2 and CD8 negativity and CD1a, CD4, CD5, CD7 and CD10 positivity. Four inv(7) positive cases were analyzed for CD13 and CD33 expression and were found to be negative.

Cytogenetic analysis showed abnormal karyotypes in 11 out of 19 *TCRβ* positive cases (Table 1). Translocations affecting the 7q34 locus were detected upon banding analysis in three of 19 cases and partial deletions of chromosome 7q or 7p in three cases; one case showed additional material on 7q. Altogether, *TCRβ* genomic rearrangement was unsuspected from

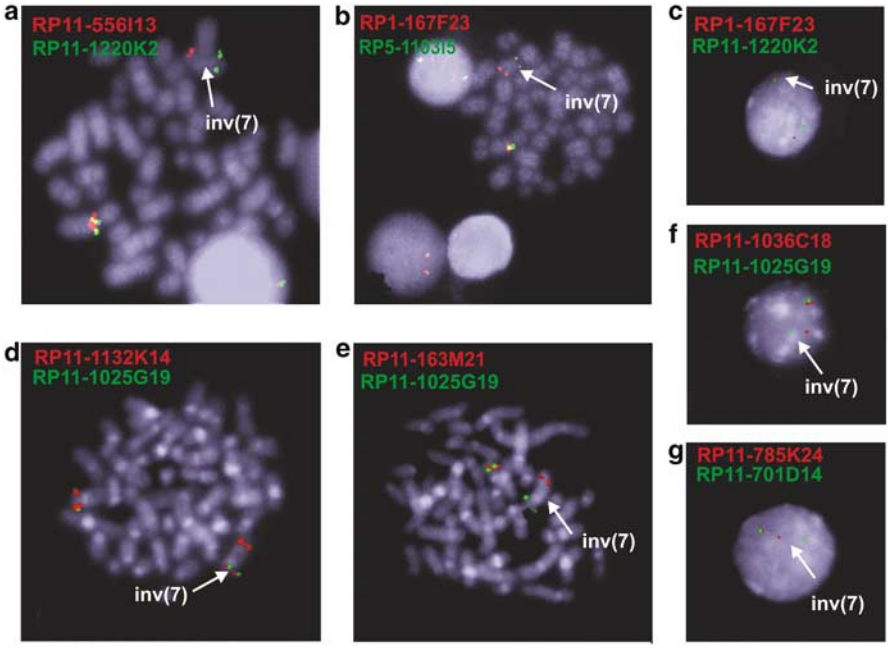

Figure 1 Dual colour FISH results using (a) *TCRβ* flanking probes RP11-1220K2 (green) and RP11-556I13 (red) demonstrating disruption of the *TCRβ* locus due to the inv(7) (case no. 18); (b) *HOXA* flanking centromeric probe RP5-1103I5 (green) with the telomeric and partly covering probe RP1-167F23 (red) demonstrating disruption of the *HOXA* gene cluster due to the inv(7) (case no. 18); (c) centromeric flanking *TCRβ* probe RP11-1220K2 (green) with the telomeric *HOXA* flanking probe RP1-167F23 (red), which yielded the expected fusion signals on the inverted chromosome 7 in interphase nuclei (case no. 17); (d) *HOXA* covering probes RP11-1132K14 (red) and RP11-1025G19 (green) revealing two fusion signals and an extra red signal (case no. 16); (e) *HOXA* covering probes RP11-163M21(red) and RP11-1025G19 (green) yielding one fusion and one split signal (case no. 16); (f) *HOXA* covering probes RP11-1036C19(red) and RP11-1025G16(green) yielding one fusion and one split signal(case no. 16); (g) *TCRβ* covering clones RP11-785K24 (red) and RP11-701D14 (green) gave the expected split signals in inv(7) (case no. 17).

Table 2 Immunophenotype of 30 T-ALL patients

Case no.	*1*	*2*	*4*	*5*	*6*	*7*	*8*	*9*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*
CD34	60	2	0	0	1	0	0	54	nd	0	0	56	3	0	0	0	nd	0	0	0	88	1	0	0	3	0	3	31	0	82
cyCD3	90	99	99	nd	99	49	82	100	nd	95	nd	92	98	94	86	nd	nd	24	91	98	90	59	99	98	76	99	nd	89	88	37
CD2	86	99	100	73	99	96	50	91	0	100	100	79	2	6	0	26w	9	96	99	17	1	97	99	99	75	0	nd	92	99	13
CD7	86	99	99	70	99	95	99	87	99	97	97	95	96	73	98	99	72	96	99	97	98	94	99	99	88	99	nd	92	98	92
CD5	88	99	99	76	15	96	97	92	96	98	99	96	96	74	91	98	94	95	99	87	86	96	99	99	43	80	nd	92	98	72
CD1a	38	24	98	56	88	0	0	5	50w	98	97	40	75	41	43	98	5	94	0	0	0	2	21	99	14w	60	nd	29	0	0
CD3m	80	95	99	98	95	23	98	96	59	1	2	1	3	73	85w	98	73	3	0	97	0	21	98	0	50w	83	3	60	96	0
CD4	22	56	99	13	1	36	95	3	50w	0	98	20	93	58	43	75	48	95	0	1	0	92	80	100	54	76	0	0	69	0
CD8	12	96	99	76	99	74	96	5	84	99	34	91	1	15	0	1	3	50	0	1	0	95	90	100	15	15	95	60	86	0
CD10	0	2	99	74	99	16	0	0	97	96	1	94	95	69	73	70	92	50	99	0	11	9	20	99	0	37	98	73	34	77
TCRgd	0	0	0	2	1	0	0	0	57w	0	nd	nd	2	56	0	nd	1	0	0	87	0	1	0	0	0	45	nd	2	0	0
TCRab	0	93	49	3	1	0	0	99	0	0	nd	nd	0	5	0	nd	6	3	0	3	0	20	97	99	0	0	nd	8	0	0
EGIL	T4	T4	T4	T4	T4	T4	T4	T4	T4	T3	T3	T3	T3	T4	T4	T4	T4	T3	T2	T4	T2	T4	T4	T3	T4	T4	?	T4	T4	T2
TCRβ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−

Data represent % of blast cells positive for the respective antigen (CD); nd: not done; w: weak.
TCRβ genomic rearrangement: +: present; −: absent.
Case numbers correspond to those in table n°1.
Antigens are arranged in order in which they appear during T-cell differentiation.

cytogenetic analysis in 16/19 cases, partly due to inferior quality of metaphase chromosomes in T-ALL. Details on cytogenetic findings for all 94 cases including additional FISH analysis for the other *TCR* loci will be reported elsewhere (Cauwelier *et al*, in preparation).

Inv(7)(p15q34) is a new recurrent rearrangement in T-ALL

Since the *HOXA* gene cluster is known to be involved in normal human T-cell development,[13] this locus was considered as candidate partner gene for rearrangement with the *TCRβ* locus. Therefore, dual color hybridization was performed on the inv(7) positive cases using probes flanking the *HOXA* gene cluster located at 7p15 (RP1-167F23 telomeric and RP5-1103I5 centromeric clones). In all five inv(7) positive cases, a split for the *HOXA* flanking probes was observed, with the telomeric clone being inverted to distal 7q (Figure 1b). To confirm these results, we combined the centromeric flanking *TCRβ* probe RP11-1220K2 with the telomeric *HOXA* flanking probe RP1-167F23, which yielded the expected fusion signals on the inverted chromosome 7 in metaphases as well as in interphase nuclei (Figure 1c).

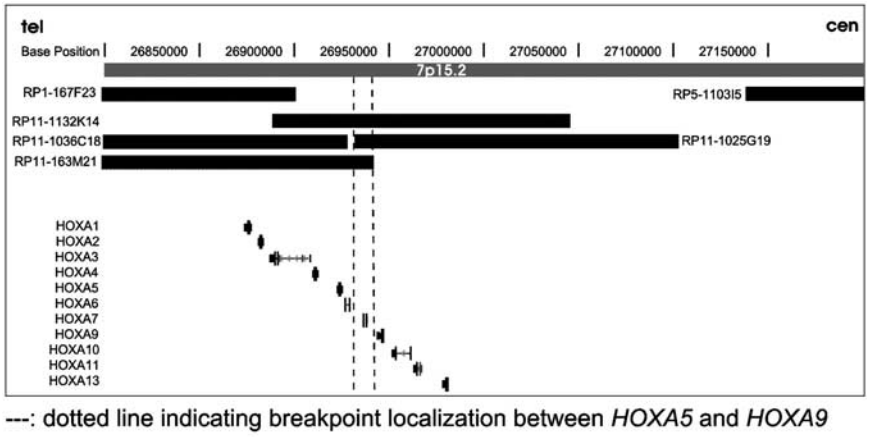

Figure 2 Base position of *HOXA* flanking (RP11-167F23 telomeric and RP11-1103I5 centromeric) and *HOXA* covering clones (RP11-1132K14, RP11-1036C18, RP11-163M21 and RP11-1025G19), according to UCSC Genome Browser May 2004 Freeze.

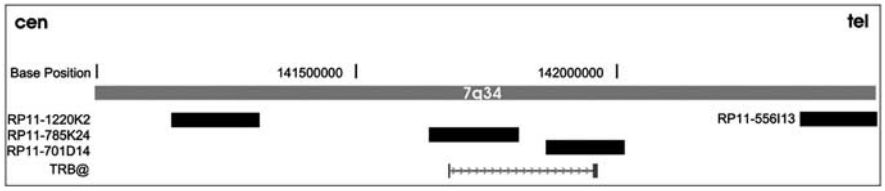

Figure 3 Base position of *TCRβ* flanking (RP11-1220K2 centromeric and RP11-556I13 telomeric) and covering clones (RP11-785K24, RP11-701D14), according to UCSC Genome Browser May 2004 Freeze.

Further characterization of the chromosome 7p breakpoint using three different probe combinations covering the *HOXA* locus (Figure 2) yielded similar hybridization patterns in all five inv(7) positive cases. For each of the three probe combinations (centromeric clone RP11-1025G19 combined with more telomeric located clones RP11-1036C18, RP11-163M21 or RP11-1132K14, respectively) split signals of the combined probes and complete (RP11-1036C18, RP11-163M21) or partial (RP11-1132K14) inversion of the more telomeric located probes were observed (Figure 1d–f). Consequently, these experiments further confirmed the localization of the inversion 7p15 breakpoint within the *HOXA* gene cluster and more specifically allowed us to assign the breakpoint to the region covered by BAC clone RP11-1132K14. Given the relative position of the four BAC clones used in these experiments and absence of disruption of RP11-1036C18, RP11-163M21 or RP11-1025G19, we assume that the most probable position of the 7p15 breakpoint is within a $\sim$20 kb segment located between *HOXA5* and *HOXA9* (Figure 2).

Two clones spanning most of the *TCRβ* locus (RP11-785K24 centromeric and RP11-701D14 telomeric) revealed split signals in all inv(7) positive cases (Figure 1g), and thus confirmed the involvement of the *TCRβ* gene in the inv(7) positive cases (Figure 3). Moreover, in two inv(7) positive cases a partial deletion of the centromeric *TCRβ* probe in 95% (case no. 15) and 96% of cells (case no. 18) carrying the inversion was observed.

Real time quantitation of gene expression for HOXA, HOXB3 *and* HOXC4 *genes*

Expression level measurements of all eleven members of the *HOXA* gene cluster *(HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11 and HOXA13)* were performed on a total of 26 patient samples, total CT and CD34+ purified child thymocytes (CD34+T). Patient samples included five inv(7) positive cases (group A), eight *TCRβ* positive without inv(7) (group B) and 13 *TCRβ* negative selected cases (group C). As no significant differences in the observed expression pattern was noted for group B as compared to group C (Student's *t*-test: $P > 0.05$), both patient groups were joined and inv(7) positive cases were compared to inv (7) negative cases. A high *HOXA10* and *HOXA11* expression was noted for all inv(7) positive cases whereas two inv(7) negative T-ALLs showed similar increased levels of *HOXA10* and *HOXA11* transcripts (cases no. 21 and 22) (Figure 4A, B). Levels of *HOXA10* and *HOXA11* expression in total CT and CD34+ purified child thymocytes were comparable but at least 10-fold lower than the mean expression of these genes in inv(7) positive T-ALL samples. *HOXA11* expression levels were statistically significant increased in the inv(7) positive cases *vs* inv(7) negative cases ($P = 4.5 \times 10\text{E-}4$ and $P = 8.8 \times 10\text{E-}4$, respectively), the latter showing only marginal expression for *HOXA10* and *HOXA11*. *HOXA9* expression was slightly increased in inv(7) positive patients as compared to the inv(7) negative samples ($P = 1.8 \times 10\text{E-}3$). Remarkably, in one inv(7) positive case (no. 15), all other *HOXA* genes also showed increased expression (Figure 4C(a) and (b)). Likewise, one inv(7) negative case (no. 28) showed elevated expression for all *HOXA* genes except for *HOXA11* (Figure 4C(a) and (b)). For these three cases showing elevated expression for *HOXA10* and/or *HOXA11* (cases no. 21, no. 22, no. 28), dual color FISH using the *HOXA* flanking probes was performed to evaluate disruption of the *HOXA* gene cluster but no evidence for any disruption of the *HOXA* gene cluster was found.

In addition to *HOXA* gene expression, we tested the inv(7) positive patients, total CT cells and CD34+ purified child thymocytes (CD34+T) for expression of *HOXB3* and *HOXC4*

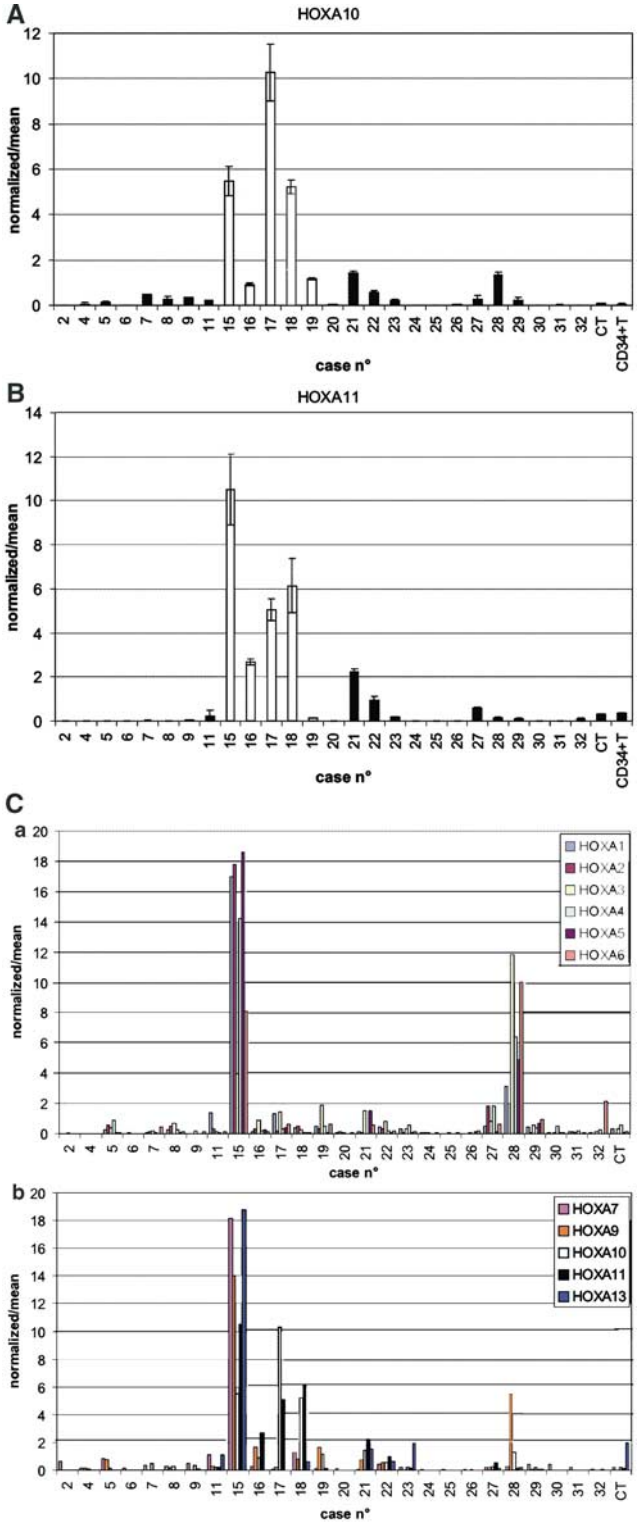

Figure 4 (A) Geometric averages of normalized expression of *HOXA10* in inv(7) positive T-ALL cases (white boxes), inv(7) negative T-ALL cases (black boxes), total child thymocytes (CT) and CD34+ sorted stem cells (CD34+T). (B) Geometric averages of normalized expression of *HOXA11* in inv(7) positive T-ALL cases (white boxes), inv(7) negative T-ALL cases (black boxes), total child thymocytes (CT) and CD34+ sorted stem cells (CD34+T). (C (a and b)) Global overview of normalized expression of all class I *HOX* genes tested in inv(7) positive T-ALLs (cases no. 15–19), inv(7) negative T-ALLs (cases no. 2–11 and no. 20–32) and total child thymocytes (CT).

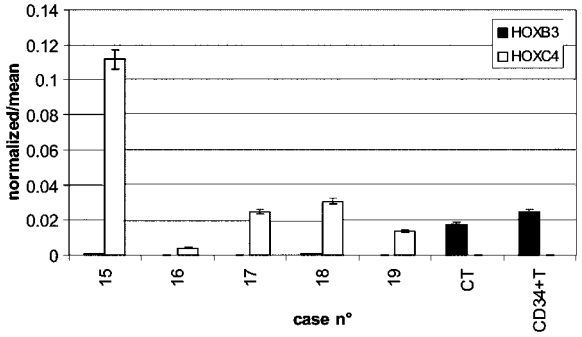

Figure 5 Geometric averages of normalized expression of *HOXB3* and *HOXC4* in inv(7) positive T-ALL cases (no. 15–19), total child thymocytes (CT) and CD34+ sorted stem cells (CD34+T).

gene expression since, according to Taghon *et al*,[13] these were the only homeobox genes apart from *HOXA7, HOXA9, HOXA10* and *HOXA11* that were expressed at all major stages of human thymopoiesis. In comparison with child thymocytes and CD34+ purified child thymocytes, *HOXB3* was only weakly expressed in the inv(7) positive patients whereas *HOXC4* expression was clearly detected in all inv(7) positive patients and slightly higher than expression levels of total child thymocytes and CD34 purified child thymocytes (Figure 5).

Discussion

In this study, we describe a new recurrent chromosomal alteration, a pericentric inversion of chromosome 7, inv(7)(p15q34), in five patients with T-ALL leading to disruption of the *HOXA* gene cluster and juxtaposition to sequences within the *TCRβ* locus. As a consequence of this rearrangement, increased *HOXA10* and *HOXA11* expression was evident in all inv(7)(p15q34) positive cases as compared to most inv(7) negative T-ALLs.

A number of findings support our assumption that this inv(7)(p15q34) indeed represents a new recurrent chromosomal abnormality in T-ALL. First of all, FISH results with BAC clones covering the *HOXA* gene cluster at 7p15 were similar in all cases and were in keeping with a breakpoint cluster within the ~107 kb genomic region containing all *HOXA* genes, most probably distal to *HOXA5* and proximal to *HOXA10*. Second, gene expression analysis showed consistent increased expression of *HOXA10* and *HOXA11* in all inv(7) positive cases. Thirdly, all inv(7) positive T-ALLs were classified as mature T-ALLs (T3–4 group according to the EGIL recommendations[10]) but with characteristic CD2 negative CD4 positive, CD8 negative blast cells.

The present study was initially triggered by the finding of a cryptic t(7;11)(q34;q24) in an M-FISH study of childhood ALLs with complex karyotypic rearrangements. Based upon this observation, we anticipated that the distal chromosomal localization of the *TCRβ* locus might also predispose to the formation of other undetected rearrangements, which indeed turned out to be the case as demonstrated by the finding of the recurrent inv(7)(p15q34). In addition to this new discovery, the finding of chromosomal rearrangements affecting the *TCRβ* locus in as much as 20% in an unselected group of T-ALLs is in itself remarkable. Although the study by Gesk *et al*[8] showed a relatively high proportion of *TCRβ* rearrangements in a series of T-ALLs with cytogenetically detectable rearrangements affecting

the *TCR* loci, no systematic study on unselected cases investigating *TCRβ* rearrangements has been reported thus far. The high percentage of *TCRβ* rearrangements is only partly explained by the presence of the five inv(7) cases. Indeed, for 8/19 *TCRβ* positive cases, FISH screening for known *TCRβ* partners was negative and further testing for partner identification is currently ongoing. In addition, some chromosomal disruptions of the *TCRβ* locus were found in patients with apparent normal karyotypes and representing partly cryptic rearrangements with known T-cell oncogenes such as *RBTN2* and *RBTN1* with subtelomeric localization.

Homeobox genes are a particular class of transcription factors that play an important role in regulating aspects of morphogenesis and cell differentiation during normal embryonic development. Class I *HOX* genes are located in tightly linked physical clusters, whereas class II *HO*X genes are dispersed throughout the genome. In humans, a total of 39 class I *HOX* genes are located in four distinct gene clusters on 7p15.3 (*HOXA*), 17q21.3 (*HOXB*), 12q13.3 (*HOXC)* and 2q31.1 (*HOXD).*[19] They encode a complex network of transcription regulatory proteins whose precise targets remain poorly understood. *HOX* genes were shown to play a significant role not only in regulating body formation but also in organization and regulation of hematopoiesis[20] and leukemogenesis.[21–23] Direct effects of class I *HOX* gene function in hematopoiesis have been shown in various studies where *HOX* gene expression has been modulated by antisense oligonucleotides,[24,25] gene disruption by homologous recombination[26,27] or by overexpression studies.[28–31] In brief, forced expression of *HOXA10* in murine[31,32] and cord blood[28,29] hematopoietic stem cells resulted in increased proliferation and impaired myeloid differentiation besides a profound reduction in B and T cell differentiation. *HOXA9* overexpression studies showed similar effects in myeloid lineages but without effect on T lymphoid development.[33] Both overexpression studies of *HOXA10* and *HOXA9* however failed to produce any lymphoid neoplasms. In myeloid leukemia, several class I *HOX* genes have been implicated predominantly due to formation of fusion transcripts with *NUP98*.[34] A role for class I homeobox genes in T-ALL was first suggested by Ferrando *et al*,[35] who demonstrated consistently increased expression levels of a subset of *HOX* genes (*HOXA9, HOXA10, HOXC6* and *MEIS1*) in *MLL* rearranged T- and B-ALL, while myeloid lineage genes were only expressed in *MLL* rearranged B-ALL and not in T-ALL cases. These findings suggested *HOX* gene deregulation rather than myeloid gene deregulation being the key factor in leukaemic transformation mediated by *MLL* fusion proteins.[35] Further support for a crucial role of *HOXA* genes came from a study of *HOXA* gene expression in developing thymocytes[13] where high *HOXA10* expression was found in the earliest T-cell precursors in human thymus, which showed subsequent downregulation in CD4 and CD8 single positive mature thymocytes. This observation suggested a role for *HOXA10* in T-cell maturation, which was in keeping with their previous studies that showed impairment of final T-cell differentiation upon enforced *HOXA10* expression in cord blood cells.[29] In contrast to *HOXA* genes, expression of the other *HOX* cluster genes was less prominent or absent except for *HOXB3* and *HOXC4. HOXA11* was expressed at all major stages of T-cell development also underlining the putative important role of this particular *HOX* gene in thymocyte maturation. Taken together, the present findings and published data on *HOXA* expression in T-cell precursors and maturing thymocytes strongly suggest that the *HOXA* gene cluster and *HOXA10* and *HOXA11* genes, in particular, are of crucial importance in normal T-cell development.

In contrast to class I homeobox genes, two particular class II homeobox genes has been extensively studied in T-ALL, that is *HOX11*, initially discovered through the t(10;14)(q24;q11),[36] and *HOX11L2* more recently shown to be implicated in the cryptic t(5;14)(q35;q32).[37]

Chromosomal rearrangements disrupting *HOX* gene clusters might also shed light onto the mechanisms controlling *HOX* gene expression, that is, locally cis-acting control sequences, global enhancer sequences located outside the gene clusters.[38,39] Here, we propose two possible oncogenetic mechanisms resulting from the inv(7). First, the disruption of the *HOXA* gene cluster could interfere with the normal scheduled program of sequential up and downregulation following the 3′ to 5′ localization of the *HOXA* genes, that is, 3′ region *HOXA* genes being expressed in the more primitive cells and 5′ genes in more differentiated cells. The physical disruption of the cluster could therefore block downregulation of *HOXA10* and *HOXA11* required in order to allow the cells to complete their further differentiation and maturation. The second and more classical hypothesis is that enhancers embedded within the *TCRβ* locus lead to sustained high expression levels of *HOXA10* and *HOXA11*. Based upon the 10-fold higher expression levels observed for *HOXA10* and *HOXA11* in the inv(7) positive cases as compared to normal developing thymocytes, we favor the latter hypothesis.

Immunophenotypically, the five inv(7) patients showed a distinct pattern of CD2 negativity and CD4 single positivity within the T3–4 subgroup of T-ALLs. Obviously, lack of CD2 expression on their leukaemic blasts is the most striking feature as this pan T-cell marker is present on the leukaemic blasts of 85% of T-ALL.[40] CD4 single positivity is another intriguing finding within the T3–4 group as most cases either show CD4/CD8 double positivity or CD8 single positivity. Taken together, the phenotypic combination of CD2−, CD4+ and CD8− showed significant correlation with the presence of the inv(7) genomic rearrangement ($P = 5 \times 10E\text{-}5$).

In addition to the inv(7)(p15q34) positive cases, our study indicates that other mechanisms can lead to abnormal *HOXA* gene expression as two inv(7) negative cases also showed an elevated *HOXA10* and *HOXA11* expression. Moreover, a third case was noted, which like one of the inv(7) positive cases, showed upregulated expression of all *HOXA* genes. This observation is intriguing and somewhat reminiscent to the observed enhanced expression of certain T-cell oncogenes *(HOX11, LYL1, TAL1, LMO1 and LMO2)* in the absence of any detectable chromosomal rearrangement affecting these loci.[41] Further study of these remarkable cases might increase our understanding of the mechanisms governing the tight control of expression of these developmentally important genes.

In conclusion, the present report describes a new recurrent cryptic chromosomal inversion inv(7)(p15q34) in T-ALL,which leads to elevated expression of *HOXA10* and *HOXA11* in five cases and increased expression of all *HOXA* genes in one case and seems to delineate a specific subset of CD2 negative CD4 single positive T-ALLs. This observation for the second time implicates class I *HOXA* genes, and in particular *HOXA10* and *HOXA11*, in T-cell oncogenesis and strongly supports the previously proposed role for *HOXA* genes in thymocyte development. We are currently collecting additional cases of inv(7) patients in order to determine in more detail the clinical and biological profile of this patient subgroup and, most importantly, in order to assess the prognostic importance of inv(7)(p15q34) within a large cohort of uniformly treated patients. Finally, we anticipate that this study might trigger a search for compounds interfering with T-cell differentiation

pathways controlled by *HOXA* genes and will also stimulate new investigations focused at determining the role of *HOXA* genes in T-cell development.

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