T Cell Receptor Genotyping and HOXA/TLX1 Expression Define Three T Lymphoblastic Lymphoma Subsets which Might Affect Clinical Outcome

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Abstract

Purpose: T lymphoblastic lymphomas (T-LBL) are rare disorders of immature T cells which predominantly involve the mediastinum. Their oncogenic pathways and prognostic variables are not clear.

Experimental Design: We undertook a retrospective study of 41 cytoplasmic CD3+ T-LBL (nine cases aged 116 years) by assessing stage of maturation arrest based on T cell receptor (TCR) immunogenotyping, immunohistochemistry, and quantification of the oncogenes thought to be important in immature T cell malignancies.

Results: Application of a TCR-based immunogenetic classification allowed the identification of three subcategories: 11 immature IM0/D-LBL showed no TCR or only incomplete TCRD DJ rearrangement and corresponded to cytoplasmic CD3+ precursors of uncertain lineage. Sixteen mature TCRDdel-LBL showed biallelic TCRD deletion and both TCRG and TCRB rearrangement, consistent with TCRβ1 lineage restriction. Fourteen intermediate LBL (Int-LBL) showed complete TCRD VDJ and TCRG VJ rearrangement, with TCRB VDJ rearrangement in the majority. All Int-LBL expressed HOX11/TLX1 or HOXA9 transcripts and a proportion of the latter were associated with CALM-AF10 or NUP214-ABL fusion transcripts. IM0/D-LBL were restricted to adults with extrathyroidal disease and bone marrow involvement, whereas Int-LBL and TCRDdel-LBL were found in children and adults with predominantly thymic disease. In adults, the Int-LBL subgroup was associated with a significantly superior clinical outcome. This subgroup can be identified either by TCR immunogenotyping or HOXA9/TLX1 transcript quantification.

Conclusion: Application of this molecular classification will allow the prospective evaluation of prognostic effects within pediatric and adult protocols.

T lymphoblastic lymphomas (T-LBL) are rare non–Hodgkin lymphomas defined by the expansion of immature cytoplasmic CD3 (cCD3) expressing lymphoblasts, frequent mediastinal involvement, and absent or <25% bone marrow involvement (1). They represent 2% of adult and 20% of pediatric non–Hodgkin lymphomas, with an overall incidence of ~0.1 per 100,000 inhabitants/y, and predominantly occur in male adolescents or young adults (2, 3). The use of T-acute lymphoblastic leukemia (ALL)–derived protocols has improved the prognosis, which nevertheless, remains strikingly different in adults and children, with 5-year overall survival rates of ~55% and 90%, respectively (2, 4). Although the Revised European-American Lymphoma/WHO classification of lymphomas provides morphologic and phenotypic features useful for T-LBL diagnosis (1), little data is available regarding the clinical or biological prognostic factors in T-LBL, particularly for adults. Analysis of lymphoma is often limited by the absence of appropriate material for flow cytometric and transcriptional analyses. We have therefore adapted a T cell receptor (TCR)–based classification of T-ALL (5) to a molecular T-LBL classification, which allowed us to identify subsets of patients with distinct immunophenotypes and clinical outcomes.
During normal thymic development, TCRαβ expression is preceded by the pre-TCR, composed of TCRβ complexed to an invariant protein, pTα (6). This induces a massive proliferative and survival process known as beta-selection (6). Entry into beta-selection occurs immediately after completion of TCRB VDJ rearrangement. The order of TCR rearrangement is strictly coordinated, starting with TCRD DD and DJ, followed by TCRB VDJ rearrangement (7). TCRα rearrangement and consequent TCRD deletion, usually on both alleles, occurs after beta-selection (6). Biallelic TCRD deletion in the presence of TCRB VDJ rearrangement therefore indicates a TCRαβ lineage population, although a minority of mature TCRαβ cells and 37% of TCRαβ T-ALLs (8) retain one TCRD allele. Mature TCRδ cells have rearranged TCRD VDJ and TCRG VJ, but could also show in-frame TCRB VDJ in conjunction with a surface TCRγδ (9–11). The detection of both TCRB and TCRD VDJ rearrangements is therefore compatible with both the TCRαβ and TCRδγ linesages.

We classified cCD3+, CD7+ T-ALL into three groups: mature T-ALL, which express a surface (s) TCRαβ or TCRγδ; pre-αβ T-ALLs, which are cCD3+, cTCRγδ+; and immature (IM) cTCRαβ- T-ALL, which were further subdivided into IM0 (no rearrangement), IMD (TCRB+Gβδ), IMG (TCRD+Gδβδ), and IMB (TCRD+Gβγδ). IM-T-ALLs and TCR+ T-ALLs are overrepresented in adults and children, respectively (5). The recognized, “classical”, T-ALL oncogenes, SITL-TALI, TLX3/HOX11L2, and TLX1/HOX11 are restricted to αβ-lineage T-ALLs (IMB, pre-αβ, and TCRαβ+), despite the expression of unusual TCRγδ receptors by a minority of TLX3+ cases. CALM-AF10 and MILL rearrangements are restricted to γδ-lineage T-ALLs with an IMD/G or TCRγδ stage of maturation arrest, and occur in ~30% and 10% of cases, respectively (12, 13). As for SITL-TALI fusions, idiopathic SCL/TAL1 overexpression predominates in γδ-lineage T-ALLs, whereas LMO2 and LYL1 expression is most common in IM T-ALLs, in which they often coexist (14, 15). The expression of HOXA cluster genes, particularly HOXA9, has been described in association with the translocation of TCRB or TCRD into the HOXA cluster (16–18). HOXA expression in T-ALL can also be associated with MIL (19) or CALM-AF10 rearrangement (20), but has also been described in isolation (16).

In the present study, we show that T-LBL can be divided into three groups: immature, intermediate HOXA/TLX1 - expressing, and mature TCRδε T-LBLs, with distinct clinicobiological features and outcomes. Prospective application of this molecular classification will orientate biological analyses designed to identify additional underlying oncogenic mechanisms and prognostic subgroups in pediatric and adult T-LBL.

### Materials and Methods

**Patients and clinical presentation.** Forty-one cryopreserved diagnostic samples (25 lymph nodes, 10 mediastinal biopsies, and 6 pleural effusions) from 9 pediatric cases with a median age of 10 years (range, 2-15 years) and 32 adolescents or adults, with a median age of 31 years (16-70 years) were collected from eight clinical centers. All biopsies had been collected with informed patient consent. All were massively infiltrated by cCD3+, CD19, and/or CD20-negative small- to medium-sized blasts. When present, bone marrow involvement was <25%, as assessed morphologically. The male/female ratio was 3:1 in adults, whereas there was a disproportionate number of female cases in the small pediatric series (Table 1).

Clinical records and computerized tomography scans were available for independent review, without knowledge of the biological data, for 30 of the 41 patients. It was possible to distinguish two distinct presentations: T-LBL with a bulky mass of thymic origin and lymph node–based T-LBL, which often included mediastinal lymph nodes, with dissemination to peripheral or retroperitoneal lymph nodes, but with no intrinsic thymic mass. Thymic-based T-LBL corresponded to 63% of cases overall and continued to occur in adults >50 years old. Central nervous system involvement was found in 9% of adults but not in the nine pediatric cases. Bone marrow involvement was identified in 45% of adults and a single pediatric case (one of the two with lymph node–based disease). Clinical outcome was available for 28 patients (20 aged 16 years or over) with a median follow-up of 4 years. In keeping with the different strategies reported, the treatment was variable in this retrospective series (2, 21–23). Of the 20 adult patients with clinical follow-up available, 19 were treated on or according to leukemia or lymphoma protocols, as detailed in Supplementary Table S1. These included seven GELA87/93, four LALA94, three GRAALL03/pilot, three FRALLE93/2000, and two Société Française d’Oncologie Pédiatrique (SOF) Lymphome T (LMT) 89/96. Among the eight pediatric patients with clinical follow-up available, five patients were included within the LMT 96 trial (24) and three within the European Organization for Research and Treatment of Cancer 58951 trial. Overall, seven patients underwent allogeneic and one autologous transplantation after CR1 was achieved.

**Immunohistochemistry and antibodies.** As inclusion criteria, all cases were cCD3-positive and CD19- or CD20-negative. Immunohistochemical staining was done on formalin-fixed, paraffin-embedded 4-μm tissue sections by tissue microarray analysis (25). Tissue array samples (0.6 mm diameter) were punched using a manual Tissue Arrayer (Beecher Instruments), and arrayed in duplicate or triplicate (50 cores/
block). Slides were deparaffinized and pretreated in CC1 buffer (basic pH) for 68 min at 95°C. All further steps were done at 37°C on a Ventana Benchmark automated immunostainer (Ventana Medical Systems, S.A.). After blocking endogenous peroxidase activity with 3% aqueous H2O2 for 4 min (3,3′-diaminobenzidine detection kit; Ventana), the sections were incubated separately with primary antibodies: CD7 (1:100, monoclonal, clone 272; NCL-CD7-272; Novoceastra), CD2 (1:150, mouse monoclonal, clone AB75; NCL-CD2-271; Novoceastra), CD5 (1:20, mouse monoclonal, clone 4C7; NCL-CDS-4C7; Novoceastra), CD1a (prediluted, mouse monoclonal, clone O10; 1590; Immunotech), CD10 (1:40, mouse monoclonal, clone 56C6; NCL-CD10-270; Novoceastra), CD4 (1:40, mouse monoclonal, clone 4B12; NCL-CD4-368; Novoceastra), CD8 (1:50, mouse monoclonal, clone C8/1448; M7103; Dako), CD56 (1:50, mouse monoclonal, clone 186; NCL-CD56-186; Novoceastra), CD34 (1:150, mouse monoclonal, clone QBend10; Immunotech). CD117 (1:200, rabbit polyclonal, clone 4B1; NCL-CD117-1B6; Novocastra), CD3 (1:40, mouse monoclonal, clone 4C5; Novocastra), CD1a (prediluted, mouse monoclonal, clone 010; 1590; Immunotech), CD10 (1:40, mouse monoclonal, clone 56C6; NCL-CD10-270; Novoceastra), CD4 (1:40, mouse monoclonal, clone 4B12; NCL-CD4-368; Novoceastra), CD8 (1:50, mouse monoclonal, clone C8/1448; M7103; Dako), CD56 (1:50, mouse monoclonal, clone 186; NCL-CD56-186; Novoceastra), CD34 (1:150, mouse monoclonal, clone QBend10; Immunotech). CD117 (1:200, rabbit polyclonal, clone 104D2; Dako), TdT (1:30, rabbit polyclonal, clone HT-6; Dako). Then, they were washed, stained with avidin-biotin immunoperoxidase and counterstained with hematoxylin. Results were interpreted by four hematopathologists (T. Molina, P. Gaudard, D. Canioni, and A-V. Decouvelaere).

TCRD rearrangements. It is possible to detect the majority of recognized human TCRD VDJ/VDJ/VDJ rearrangements by PCR from DNA (8, 26) but TCRD deletion, atypical V(D)J such as Vα-Jα and translocations involving TCRD J segments can only be detected by Southern blotting. Such Southern+, PCR cases are collectively referred to here as atypical rearrangements. DNA was extracted from cryopreserved tissue samples or cells conserved in DMSO using Nucleon Kits (Amersham Bioscience). TCRD, TCRG, and TCRB multiplex PCR were done as previously described (ref. 26; sensitivity 1-10%), with the addition of a Vα8 upstream primer (CAgCCAAATCCTTCAgTCTCAA) to the TCRD multiplex PCR. TCRD rearrangements were also assessed by Southern blotting. Ten micrograms of DNA were digested with EcoRI and BglII, and hybridized sequentially with 32P-labeled Jα6, Jα6, and Jα probes (27). TCRD deletion was based on loss of Jα1 and Jα2 signals.

Oncogenic screening. cDNA synthesis and real-time reverse transcription quantitative-PCR (RQ-PCR) were done using European Against Cancer conditions (28). RNA and cDNA quality was assessed by cDNA synthesis and real-time reverse transcription quantitative-PCR. Amplification efficiency, primers, and housekeeping gene on an ABI PRISM 7700 (Applied Biosystems) and samples with Ct values >30 (threshold, 0.1) were considered uninterpretable. Amplification efficiency, primers, and probes used for LMO1, LMO2, TLX1, and TAL1 transcripts were as previously reported (5, 12). The NUP214-ABL fusion was detected by multiplex RQ-PCR and confirmed by monoplex RQ-PCR. NUP214 forward primers were ATggCCATgCCgCACCACCA, CACCACATTCCAgCTgCC, and TgggACCCAgcAggTAgcgg for exons 23, 29, 31, and 34, respectively, with an ABL1 reverse primer CACCTgACCCCTgAggCTCATA and probe Fam-CCCTTgCcCCgCATTCTgA-TgCTgA-Tamra. TLX1, TLX3, and NUP214-ABL overexpression was expressed as transcript/ABL ratios, with the SIL-ALL and HPB-ALL cell lines being used as positive controls for TLX1 and TLX3, respectively, and the SIL-ALL and PEER cell lines being used as positive controls for NUP214-ABL. Cutoffs for TLX1, LMO2, and TAL1 transcripts were based on the expression of each transcript by total thymocytes from 15 separate normal thymus, with cases expressing levels over the 99th percentile being considered positive. Results are expressed as transcript/ABL percentage ratios. Diagnostic screening of CALM-AF10 and SIL-TAL fusion was done as previously described (12, 29). MILL rearrangements were assessed by Southern blotting with a 32P-labeled B859 cDNA MILL probe hybridized to 10 μg of BglII or BamHI digested DNA. HOXA9 transcripts were quantified relative to ABL1 expression, as previously described (20), and were considered positive if expressed at a higher level than CALM-AF10 T-ALLs. Normal total thymocytes were obtained, with informed consent, from pediatric cases undergoing cardiac surgery. Bone marrow and peripheral blood mononuclear cells were obtained and processed as previously described (14).

Notch1 sequencing. Direct sequencing was done for the 41 T-LL samples. Exons 26 and 27, which code for the NH2- and COOH-terminal regions of the heterodimerization domain, respectively, were separately amplified by PCR in one amplicon. Exon 24 coding the PEST domain and the continuous N-region containing the TAD domain of Notch1 was PCR-amplified in three parts. Primers used were exon 26 forward AggAAcgCgCCTgAggCTgT, reverse AggAAcgCgCCTgAggCTgT, exon 27 forward gCCCTCCAgaATgAgAcgTG, reverse gcgCgCgCCTgAggCTgT, exon 24 forward CTTCCCTgAggACgCCTgT, reverse gCCCTCCAgaATgAgAcgTG, exon 34 forward CTTCCCTgAggACgCCTgT, reverse gCCCTCCAgaATgAgAcgTG, exon 34 forward CTTCCCTgAggACgCCTgT, reverse gCCCTCCAgaATgAgAcgTG. Amplification was done as previously described (12, 29).

Table 2. TCRD genotypic classification of T-LBL

<table>
<thead>
<tr>
<th>LBL subclasses (n)</th>
<th>TCRD</th>
<th>TCRG</th>
<th>TCRB</th>
<th>Southern blot TCRD (n)</th>
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<tr>
<td>IM 0/D LBL (11)</td>
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<td></td>
<td></td>
<td>GL/GL (7)</td>
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<td></td>
<td></td>
<td>R/R or R/GL (3)</td>
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<tr>
<td>&quot;IM D&quot; (3)</td>
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<td></td>
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<tr>
<td>Int-LBL (14)</td>
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<tr>
<td>&quot;IM G/GD&quot; (2)</td>
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<tr>
<td>&quot;D atypical&quot; (3)</td>
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</tr>
<tr>
<td>IMG/IM B/pre-AB/AB/GD (9)</td>
<td>VDJ</td>
<td>VJ</td>
<td>VDJ*</td>
<td>R/R or R/GL (2)</td>
</tr>
<tr>
<td>TCRD dele-LBL (16)</td>
<td></td>
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<td></td>
<td>Del/De (3)</td>
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</table>

NOTE: LBL were classified into three groups based on their TCRD, G, and B profiles using PCR and/or Southern blotting: IM0/D-LBL, Int-LBL, and TCRD dele-LBL. The number of cases for which TCRD locus have been explored by Southern blot are cited on the right. Abbreviations: IM, immature; GL, germ line; R, rearranged; Del, deleted.

*Five of six Int-LBL and three of three TCRD dele-LBL randomly sequenced show the in-frame CDR3 region.
Cases with biallelic TCRD status; they are henceforth referred to as IM0/D LBL. All 15 and as such, are likely to correspond to the mature TCR rearrangements and Int-LBL (TCRD+G+B+) corresponded to 34%, and the 16 TCRB and TCRD the 11 IM0/D-LBL (TCRD+/-G-B-) corresponded to 27%, the 14 Southern blotting. In the absence of TCR protein expression, deletion was seen in 6 of the 13 cases analyzed by PCR and/or Southern blotting in 7 cases, negative by PCR, and deleted on both alleles in 15 cases, whereas it was rearranged on at least one allele by PCR and/or Southern blotting in 16 cases. TCRG was rearranged in 30 cases and TCRB in 28 cases. The three cases with no Southern blot analyses were classified based on their TCR PCR status as IM0-LBL. However, because TCR lineage affiliation of these 14 cases is not possible and as a result, the TCR profiles in TCRDdel-LBL are similar to those seen in the majority of TCRαβ T-ALL (5), whereas those in Int-LBL are in keeping with T lymphoid orientation with a block prior to biallelic TCRα rearrangement but, no block to complete TCRD, TCRB, and TCRB rearrangement. Because complete rearrangement of the TCRD, TCRB, and TCRB loci is preferentially associated with deregulated TLX1 or TLX3 expression in T-ALL (14), we analyzed the expression of these transcripts, as well as those of the HOXA cluster.

**Results**

**TCR genotyping by PCR and Southern blotting.** DNA-based TCRγ, 6, and β genotyping by multiplex PCR, with additional specific PCR typing when necessary (5), was possible for all 41 cases, whereas Southern blot TCRD analysis was done for 38 cases (Table 2). The TCRD locus was in a germ line configuration by PCR and Southern blotting in 7 cases, negative by PCR, and deleted on both alleles in 15 cases, whereas it was rearranged on at least one allele by PCR and/or Southern blotting in 16 cases. TCRG was rearranged in 30 cases and TCRB in 28 cases. The three cases with no Southern blot analyses were classified based on their TCR PCR status as IM0-LBL (TCRD’G’B’), TCRDdel-LBL (TCRD’G’+B’), and Int-LBL (TCRD’G’+B’).

The 11 cases with no TCRG rearrangement included 8 with a TCRD’G’B’ (IM0) status and 3 with a TCRD’D/D’G’+B’ (IMD) status; they are henceforth referred to as IM0/D LBL. All 15 cases with biallelic TCRD deletion by Southern blotting, as well as the aforementioned TCRD’G’+B’-LBL, showed both TCRG rearrangements and TCRB VDJ on at least one allele, and as such, are likely to correspond to the mature TCRαβ lineage LBL. However, because TCRαβ-expressing cells can also show TCRD rearrangements, cases with biallelic TCRD deletion were classified as TCRDdel-LBL. Fourteen LBL showed TCRD and TCRG rearrangements, of which 12 also showed clonal TCRB VDJ rearrangements (TCRD’G’+B’). Monoallelic TCRD deletion was seen in 6 of the 13 cases analyzed by Southern blotting. In the absence of TCR protein expression, TCR lineage affiliation of these 14 cases is not possible and they are classified together as intermediate (Int-LBL). Overall, the 11 IM0/D-LBL (TCRD’D/D’G’+B’) corresponded to 27%, the 14 Int-LBL (TCRD’D/D’G’+B’) corresponded to 34%, and the 16 TCRDdel-LBL (TCRDdel’D/D’G’+B’) corresponded to 39% of cases.

All TCRB’+LBL showed a complete VDJ on at least one allele; cases with only TCRB DJ were strikingly absent. Of the 12 Int-T-LBL demonstrating TCRB VDJ rearrangement, 6 were randomly sequenced and 5 showed in-frame CDR3, including 1 CALM-AF10 – positive case (see below). TCRG rearrangements were predominantly biallelic Vγ1-Jγ1/2 “end-stage” rearrangements. The TCRD rearrangements in the three IMD-LBL included two D62-D63 and four D62-J61 alleles. The 13 rearranged TCRD alleles detected by PCR in 10 Int-LBL included 1 V62-D63, 2 D62-J61, and 10 V66-J61 (5 V61, 2 V63, and 1 each of V62, V65, and V68). Taken together, the TCR profiles in TCRDdel-LBL are similar to those seen in the majority of TCRαβ T-ALL (5), whereas those in Int-LBL are in keeping with T lymphoid orientation with a block prior to biallelic TCRα rearrangement, but no block to complete TCRD, TCRB, and TCRB rearrangement.

**T-LBL oncogenic transcripts.** We quantified the expression of HOXA9, HOX11/TLX1, HOX11L2/TLX3, TAL1, LMO1/2, and LYL1 transcripts by RQ-PCR and searched for the SIL-TAL, CALM-AF10, and NUP214-ABL fusion transcripts in the 37 T-LBL with available RNA (Table 3). HOX11/TLX1 was overexpressed in three Int-LBL. Three other Int-LBL showed CALM-AF10 transcripts, all of which corresponded to the long form predominantly found in TCRγ6 expressing T-ALLs (data not shown; ref. 12). HOX11L2/TLX3 or SIL-TAL expression was not seen.

The total incidence of Notch1 mutations was 22 of 41 (54%). Mutations were seen in 16 of 32 and 6 of 9 adult and pediatric cases, respectively (Table 3). The type of mutation was 32%, 32%, and 36% for HD, PEST, and HD + PEST domains, respectively.

**HOXA9** transcripts in normal tissues were identified in total thymocytes and a proportion of bone marrow samples but not in peripheral blood (Fig. 1). All three CALM-AF10+ LBL expressed HOXA9. The cutoff for HOXA9 transcript positivity in T-LBL was arbitrarily fixed at the lowest level (≥30%) observed in these cases and in 13 previously reported CALM-AF10+ T-ALL (20). Using this cutoff, HOXA9 expression was seen in 12 of 37 T-LBL (32%; Fig. 1). Expression correlated strikingly with Int-LBL, when it was seen in 9 of 12 cases tested.
and notably in all cases other than those expressing TLX1, compared with only 1 of 10 IM0-D-LBL and 1 of 15 TCRDdel-LBL (Fisher exact test, \( P < 0.001 \) for Int-LBL versus non-Int-LBL). MLL rearrangements were not seen by Southern blot in the eight HOXA9+ CALM-AF10–negative Int-LBL analyzed (data not shown). A NUP214-ABL transcript of identical size to those identified in T-ALL was detected in one Int-LBL. In T-ALL, NUP214-ABL is usually associated with TLX1 or TLX3 expression (30). The present case expressed neither but did express HOXA9 (Fig. 1).

**TAL1** expression was rare (Table 3; Supplemental Fig. S1A), being restricted to sporadic cases which coexpressed LMO1 (one case with TCRDdel-LBL) or LMO2 (three cases with IM0/D-LBL). LMO1 expression was restricted to TCRDdel-LBL. LMO2 and LYL1 were significantly more frequently expressed in IM0/D-LBL (Supplemental Fig. S1B and C) compared with Int-LBL. 9 of 11 CD4/8DP compared with 0 of 7 CD8SP/DN LBL. The LMO1 expression (30). The present case expressed neither but did express HOXA9 and HOXA10 (Fisher exact test, \( P = 0.005 \) for LMO2 and \( P = 0.002 \) for LYL1).

Taken together, the predominant transcripts identified were HOXA9 (32%) or TLX1 (8%), with the former being associated with CALM-AF10 or NUP214-ABL in a proportion of cases. Common oncogenic transcripts seen in pediatric T-ALL (TLX3 and SIL-TAL1) were absent in this predominantly adult series but TLX1 and LMO2 were, as for T-ALL, found preferentially in immature cases.

**T-LBL immunophenotype.** Tissue microarray analysis of 30 cases showed that CD7 was expressed by all, CD56 by none, and CD5 by 27 of 30 (Table 4). CD2 negativity was preferentially seen in IM0/D and Int-LBL categories. CD10 expression was seen in 62% overall, with no difference in the subcategories, whereas CD34 and TdT expression were more common in IM0/D-LBL. CD117+/c-kit expression was relatively constant regions were, as for T-ALL, found preferentially in Int-LBL (2 of 5 cases versus 0 of 6 IM0/D and 1 of 11 TCRDdel-LBL; data not shown).

**Clinical presentation and outcome.** The distribution between the TCR-based categories differed with age (Table 4), with IM-LBL being restricted to adults and TCRDdel-LBL tending to occur in older adults. The majority of TCRDdel-LBL and Int-LBL and all CD1a+ cases showed bulky thymic disease, whereas all IM-LBLs showed lymph node disease. Definitive bone marrow involvement was not seen in 18 thymic LBL, but was seen in 6 of 10 cases with lymph node disease (Fisher exact test, \( P < 0.001 \)). HOX11/TLX1 expression was found in three adults (25, 42, and 44 years old) with bulky thymic masses and a thymic LBL phenotype. CALM-AF10/HOX9 cases were aged 7, 22, and 23 years; two showed bone marrow involvement and one mediastinal disease. Among the five HOXA9 cases with clinical details, one had lymph node and four bulky thymic diseases. Full clinical details were not available for the 21-year-old male with NUP214-ABL LBL.

Evaluation of clinical outcome was possible in 20 adults (8 TCRDdel, 6 intermediate, and 6 IM0/D) and 8 children (4 TCRDdel, 4 intermediate). No clinical follow-up data was available for the two HOXA9+ non-Int-LBL. As detailed in Supplementary Table S1, 19 adults were treated according to national prospective ALL or non–Hodgkin lymphomas (NHL) protocols active at the time of diagnosis; including 7 on GRAALL or LALA adult ALL protocols, 3 according to FRALE pediatric protocols, 7 with GELA adult non–Hodgkin lymphoma treatment strategies, and 2 on SFOP pediatric non–Hodgkin lymphoma strategies. Three deaths were observed on the intensive ALL
chemotherapy/pediatric protocols and three deaths among those treated with NHL strategies. Two of the eight patients with transplants died. Pediatric cases were treated on European Organization for Research and Treatment of Cancer trials. The only pediatric death occurred in a TCRDdel-LBL. Given the different clinical responses in adults and children, outcome data is only given for adult cases, although a similar response was seen in pediatric cases (data not shown). No difference in clinical outcome was observed between the six IM0/D and the eight TCRDdel-LBL cases, which were consequently analyzed together. Median age in adult cases was 28.5 years (range, 16-69 years) for the IM0/D and TCRD group and 29 years (range, 22-44 years) for the Int-LBL group. Protocol distribution was similar in both groups (Supplementary Table S1). Complete remission was obtained in all pediatric and in six adult Int-LBL patients compared with only two of six IM0/D and three of eight TCRDdel-LBL. The 6 adults with Int-LBL showed a significantly superior overall survival ($P = 0.045$; Supplemental Fig. S2) compared with the 14 IM0/D and TCRDdel-LBL cases. The inclusion of pediatric data reinforced the improved overall survival in Int-LBL ($P = 0.017$) cases and gave a trend for improved event-free survival ($P = 0.05$; data not shown) because relapse and death were also seen in one of the four TCRDdel pediatric cases.

### Discussion

In order to improve our understanding of T-LBL, we describe a molecular classification which allows the identification of a relatively favorable subgroup of Int-LBL which express HOXA9 or TLX1 transcripts, compared with mature TCRa lineage LBLs with biallelic TCRD deletion, and immature IM0/D-LBLs of uncertain lineage. This classification is compared with the previously established (5, 14) T-ALL classification in Fig. 2. Evaluation of response to treatment in T-LBL has been mainly based on clinical and radiological evaluation of the kinetics of tumor mass reduction and few biological variables have been identified. It has recently been suggested that loss of heterozygosity on chromosome 6q may identify a group of pediatric patients with poor prognosis (31) and that CD94 1A expression in pediatric and adult T-LBL may identify a group with longer survival (32). We now show that T-LBL with a TCRD+G+B+/- "intermediate" genotype also represent a favorable prognostic group in a predominantly adult series. These cases can easily be distinguished from IM0/D-LBL by the detection of clonal TCRG rearrangements, but their distinction from TCRD del-LBL ideally requires Southern blot TCRD assessment, although most will be detected by the identification of clonal TCRD, TCRG, and TCRB rearrangements by PCR. Because this group can also be identified by the expression of TLX1 or HOXA9 it will be important to determine whether TCR genotype or TLX1/HOXA9 transcriptional status will most accurately identify a good prognostic subgroup. In T-ALL, TLX1 expression identifies a good prognostic subgroup (33, 34) but the prognostic effect of HOXA9 expression is not known. It was not possible to perform multivariate analyses within the present series, but the present data suggests that the value of

### Table 4. Clinical and immunohistologic characteristics of T-LBL

<table>
<thead>
<tr>
<th>Age (y)*</th>
<th>IM 0/D-LBL</th>
<th>Int-LBL</th>
<th>TCRDdel-LBL</th>
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<tr>
<td>&lt;16</td>
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<td>&gt;16</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>32</td>
</tr>
</tbody>
</table>

Median and range are only given for adult (>16 years) patients.

NOTE: The proportion of patients within a given LBL category to show thymic or lymph node–based disease, bone marrow involvement, and pediatric versus adult age of onset is shown. The majority of patients with lymph node disease were those with bone marrow involvement.

*Unlike extensive clinical data, age was available for the 41 cases.

**Only seen within the lymph node disease subclass; Fisher exact test $P < 0.001$. Antibodies used for tissue microarray immunohistologic analysis are shown with the number of cases analyzed within each LBL category indicated in parentheses. Percentages refer to the proportion of positive cases to the number of cases analyzed.
both TCR genotyping and TLX1/HOXA9 transcript quantification should be assessed, at least initially. This requires DNA and RNA of appropriate quality from fresh or cryopreserved diagnostic material. The immunophenotypic profiles identified in Int-LBL are neither sufficiently distinctive nor uniform to be used as an alternative method of identification. The availability of HOXA, TLX1, and TCRβ antibodies, which can be applied to fixed tissue, would represent a more efficient alternative to molecular diagnostics for rapid stratification but will not alter the pertinence of the availability of fresh involved tissue. The Int-LBL group was also difficult to distinguish from TCRD δ-LBL. LBL cases clinically because both subgroups were predominantly of thymic origin, did not show bone marrow involvement, and were found in both adults and children.

The relatively poor outcome of both TCRD δ-LBL and IM-LBL within adult protocols raises the question of alternative management. IM T-ALLs show an inferior response to ALL type induction within the adult LALAA94 study and the complete remission rate is lower in T-ALL (21% compared with pre-αβ, cortical T-ALLs (35). Likewise, pediatric T-ALL studies have shown a worse prognosis and resistance to some chemotherapeutic agents in immature pro-/pre–T-ALL and mature sCD3+ T-ALL (36). Conversely, the similarities between Int-LBL and pre-αβ cortical T-ALLs suggests that they could be treated on the standard risk arm of T-ALL protocols, whereas IM0/D and TCR δ-LBL should be intensified. These observations obviously need to be tested in a prospective manner.

IM0/D-LBLs were restricted to adults, show frequent bone marrow involvement, and do not seem to be of thymic origin. They may correspond to early, non–T restricted precursors, which may include very early dendritic or natural killer (NK) cell precursors (37, 38). Human NK maturation has been shown to occur in secondary lymphoid tissue, with progression from CD34+/CD117- to CD34+/CD117+ to CD34-/CD117+, NK precursors prior to CD56 expression (42). It is possible that the CD117+ LBL identified here represent malignant counterparts of these precursors. The expression of CD94 1A has also been used to distinguish potential NK-LBL from T-LBL (32), although its expression is not restricted to NK cells.

TCRD δ-LBLs were predominantly CD4/8DP or CD8SP and CD4SP was strikingly absent, as in Int-LBL. This is in contrast to T-ALL, when 27% of sTCR-ab T-ALLs are CD4SP (9% CD8SP; 50% CD4/8DP; ref. 5). This may indicate a basic helix-loop-helix–mediated influence because E2A and related basic helix-loop-helix proteins play a role in the regulation of the CD4 enhancer (43). sIL-TAL+ T-ALLs are also associated with a maturation arrest at the CD4/8DP to CD8SP but not CD4SP

The Int-LBL group was heterogeneous because it included three cases with TLX1 expression, four cases with CALM-AF10 or NUP214-ABL fusion transcripts and HOX9 expression, and five cases with idiopathic HOX9 expression. The mechanisms leading to TLX1 and HOX9 expression in CALM-AF10–negative LBL are difficult to identify in the absence of cytogenetic material. Two TLX1+ cases showed atypical TCRD rearrangements, compatible with TCRD translocations. The idiopathic HOX9 transcripts may result from deregulation in cis by translocation or other mechanisms, or from normal expression related to the stage of maturation arrest because HOX9 is expressed by immature thymocytes up to the DP stage (37, 38). MLL rearrangements were not seen but have been described in T-ALL (39). NUP214-ABL1 is usually associated with TLX1 or TLX3 expression, although two adult T-ALLs with neither have been described (40). The fact that the present case expressed HOX9 in the absence of TLX1 or TLX3 expression suggests that any potential oncogenic synergy is likely be HOX related, rather than being specific to TLX1/3. Because NUP214-ABL1 cases are appropriate candidates for imatinib therapy, these cases suggest that screening for this rare abnormality should not be restricted to TLX1/3-positive T-ALL or T-LBL. The absence of TLX3-expressing LBLs may reflect the limited number of pediatric cases studied and/or a preferential tendency to disseminate to the blood and bone marrow. In keeping with this, mediastinal involvement was relatively rare in TLX3+ adult T-ALL (35).

CD117/c-kit expression was relatively frequent in T-ALL compared with T-ALL (2% of cases; ref. 5) and was particularly common in Int-LBL, HOX9+ LBL (57%). CD117 is expressed by immature hematopoietic progenitors, NK cells, and mature mast cells (41). Human NK maturation has been shown to occur in secondary lymphoid tissue, with progression from CD34+/CD117- to CD34+/CD117+ to CD34-/CD117+, NK precursors prior to CD56 expression (42). It is possible that the CD117+ LBL identified here represent malignant counterparts of these precursors. The expression of CD94 1A has also been used to distinguish potential NK-LBL from T-LBL (32), although its expression is not restricted to NK cells.

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Fig. 2. Maturation arrest and oncogenesis-based classification of T-LBL versus T-ALL. Stage of maturation arrest is an immunophenotypic, TCR-based classification for T-ALL (5), whereas it is an immunogenetic-based classification for T-LBL. The T-ALL oncogenes are predominantly associated with the indicated T-ALL subclasses, as previously published (14). The T-LBL oncogenes are based predominantly on adult cases. *, HOX9 and TLX1 are mutually exclusive.
improved therapeutic stratification, increased interaction which could form the basis for prognostic evaluation in helix transcriptional regulator. In keeping with this, profiling of also agree with the involvement of a distinct basic helix-loop-helix transcriptional regulator. In keeping with this, profiling of human pediatric T-LBL identified distinct profiles (45).

In conclusion, we have identified three subgroups of T-LBL which could form the basis for prognostic evaluation in prospective adult and pediatric trials. This should allow improved therapeutic stratification, increased interaction between pediatric and adult protocols, and a more precise understanding of the oncogenic mechanisms in these rare disorders.

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