Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia

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Summary

Human T cell leukemias can arise from oncogenes activated by specific chromosomal translocations involving the T cell receptor genes. Here we show that five different T cell oncogenes (*HOX11*, *TAL1*, *LYL1*, *LMO1*, *and LMO2*) are often aberrantly expressed in the absence of chromosomal abnormalities. Using oligonucleotide microarrays, we identified several gene expression signatures that were indicative of leukemic arrest at specific stages of normal thymocyte development: *LYL1*$^+$ signature (pro-T), *HOX11*$^+$ (early cortical thymocyte), and *TAL1*$^+$ (late cortical thymocyte). Hierarchical clustering analysis of gene expression signatures grouped samples according to their shared oncogenic pathways and identified *HOX11L2* activation as a novel event in T cell leukemogenesis. These findings have clinical importance, since *HOX11* activation is significantly associated with a favorable prognosis, while expression of *TAL1*, *LYL1*, or, surprisingly, *HOX11L2* confers a much worse response to treatment. Our results illustrate the power of gene expression profiles to elucidate transformation pathways relevant to human leukemia.

Introduction

T cell acute lymphoblastic leukemia (T-ALL) is a malignant disease of thymocytes, accounting for 10%–15% of pediatric and 25% of adult ALL cases. Patients with T-ALL tend to present with very high circulating blast cell counts, mediastinal masses, and central nervous system involvement. The prognosis of T-ALL in children and adolescents has improved in recent years due to intensified therapies, with 5 year relapse-free survival rates now in the range of 60%–75% (Pui and Evans, 1998; Silverman et al., 2001; Chessells et al., 1995; Schrappe et al., 2000; Rivera et al., 1991). Securing further advances in treatment outcome will likely prove difficult without improved knowledge of the factors that contribute to the malignant behavior of transformed thymocytes. Unfortunately, most of the clinical and laboratory features that guide therapy for B cell precursor ALL are only marginally useful in T-ALL (Pullen et al., 1999).

Current understanding of the molecular basis of T-ALL has come largely from analysis of recurrent chromosomal translocations and intrachromosomal rearrangements. These abnormalities typically juxtapose strong promoter and enhancer elements responsible for high levels of expression of T cell receptor genes

SIGNIFICANCE

Careful analysis of clonal chromosomal abnormalities in leukemic blast cells has been a catalyst for the development of new diagnostic and therapeutic strategies. However, this line of research has had a much greater impact on the B lineage leukemias than on T cell acute lymphoblastic leukemia (T-ALL), whose pathogenesis and molecular subtypes remain largely undefined. Using a combination of DNA microarray and RT-PCR methods to analyze clinical T-ALL samples, we obtained results in support of our central hypothesis that aberrant activation of certain key transcription factor genes, often in the absence of chromosomal rearrangements, is the principal transforming event in this disease. These developmentally important molecules are shown to drive a limited number of oncogenic pathways with prognostic significance. The ability to classify T-ALL according to shared pathways of leukemic transformation has important implications for future research. It provides a conceptual framework in which to identify specific genes that determine treatment responsiveness and should foster the development of successful new therapies directed to critical molecules in pathologic transcriptional cascades. The hypothesis-driven approach to microarray analysis described in this report may also be useful for the study of other types of human cancers.

next to a small number of developmentally important transcription factor genes, including *HOX11/TLX1*, *TAL1/SCL*, *TAL2*, *LYL1*, *BHLHB1*, *LMO1*, and *LMO2* (Finger et al., 1989; Xia et al., 1991; Mellentin et al., 1989; Wang et al., 2000; McGuire et al., 1989; Royer-Pokora et al., 1991; Dube et al., 1991; Hatano et al., 1991; Fitzgerald et al., 1991; Kennedy et al., 1991; Lu et al., 1991; Brown et al., 1990; Aplan et al., 1991, 1992; Baer, 1993; Begley et al., 1989; Chen et al., 1990; Greenberg et al., 1990; Boehm et al., 1991), resulting in their aberrant expression in developing thymocytes. Although the oncogenicity of these proteins is well established (Larson et al., 1994; Neale et al., 1995; McGuire et al., 1992; Chervinsky et al., 1999; Condorelli et al., 1996; Kelliher et al., 1996; Hawley et al., 1997), understanding of the downstream transcriptional programs that generate and maintain the T-ALL phenotype remains limited. Further improvement of risk-based treatment strategies and the development of effective new drugs for T-ALL will depend on fresh insights into the molecular pathways usurped by HOX11, TAL1, and other oncoproteins in developing thymocytes.

Results

Oncogenic transcription factor expression in T-ALL

HOX11, an orphan homeobox gene essential for splenic development (Roberts et al., 1994; Dear et al., 1995), is activated in a subset of T-ALL cases bearing the t(10;14)(q24;q11) or t(7;10)(q35;q24), each of which places *HOX11* under the control of strong enhancers embedded in the T cell receptor loci. Acting on the hypothesis that *HOX11* might be aberrantly expressed in cases other than those harboring locus-specific translocations, we used quantitative real-time reverse transcriptase PCR (RT-PCR) to analyze *HOX11* expression, detecting high levels of this oncogene in 8 of 59 pediatric T-ALL samples (Figure 1A). Four of the *HOX11*-positive cases had cytogenetic abnormalities involving the *HOX11* locus in chromosome band 10q24: two t(10;14)(q24;q11.2), one t(7;10)(q35;q24), and one del(10)(q24q26). Reduced levels of *HOX11* expression (100–1000 times lower than in the eight *HOX11*$^+$ samples) were detected in four additional samples, two of which also had cytogenetic abnormalities of band 10q24 [t(10;14) and add(10)(q24)]. The remaining cases and eight normal control thymus samples showed only background levels of *HOX11* expression, near the limit of detection with this technique. Thus, by using quantitative RT-PCR analysis of the *HOX11* gene, we identified a substantial proportion of *HOX11*$^+$ cases that express high levels of the oncogene while lacking cytogenetically detectable alterations of the 10q24 region.

Prominent among T cell oncoproteins are members of the basic helix-loop-helix (bHLH) family of transcription factors: TAL1, TAL2, LYL1, and the recently described BHLHB1 protein (Bernard et al., 1990; Finger et al., 1989; Xia et al., 1991; Mellentin et al., 1989; Wang et al., 2000). These transcriptional regulators are believed to act through a common mechanism involving dominant negative interference with the activities of the E47 and E12 variants of E2A transcription factors (Begley and Green, 1999; Wang et al., 2000; Miyamoto et al., 1996; Hsu et al., 1994; Park and Sun, 1998), whose homozygous inactivation leads to T cell tumors in mice (Bain et al., 1997; Yan et al., 1997). Quantitative RT-PCR analysis revealed increased levels of *TAL1* mRNA in 29 (49%) of the 59 cases (Figure 1B). None of these samples harbored any of the recurrent translocations of chromosome band 1p32 that are known to cause aberrant expression of *TAL1* in T-ALL. However, 9 (31%) of the 29 cases with increased expression of *TAL1* (Figure 1B) had the Tal1d variant, which results from a small deletion next to the *TAL1* locus (Aplan et al., 1990; Bernard et al., 1991). Thus, the majority of cases with high levels of *TAL1* oncogene expression (22/29) lacked cytogenetic or molecular evidence of rearrangements affecting the *TAL1* locus, in agreement with results of an earlier study (Bash et al., 1995).

Thirteen (22%) of the 59 cases were classified as *LYL1*$^+$ (Figure 1C) on the basis of *LYL1* expression levels that exceeded the mean value in normal thymocytes by more than 5-fold. Increased expression of this oncogene was not associated with cytogenetic abnormalities affecting the *LYL1* locus (19p13), consistent with the paucity of reports on *LYL1* activation by chromosomal translocation. Thus, other mechanisms appear responsible for the aberrant expression of *LYL1* in thymic leukemias. Finally, *TAL2* and *BHLHB1* were expressed at high levels in a single case each (Figure 1C). Again, cytogenetic analysis failed to reveal locus-specific translocations associated with the expression of these oncogenes. Some cases overexpressed more than one of the closely related bHLH T cell oncogenes: six overexpressed both *TAL1* and *LYL1*, and one overexpressed *TAL1* and *BHLHB1*.

Analysis of the LIM-only domain genes *LMO1* and *LMO2* (Figure 1D) showed an absence of significant expression of either *LMO* gene in the *HOX11*$^+$ samples. However, overexpression of one of these genes was observed in most samples overexpressing *TAL1*, and high levels of *LMO2*, but not *LMO1*, were found in the *LYL1*$^+$ samples. These results are consistent with biochemical and transgenic animal model studies showing that LMO proteins form heterocomplexes and act in concert with TAL1 and possibly other bHLH proteins in T-ALL (Valge-Archer et al., 1994; Wadman et al., 1994; Larson et al., 1996; Chervinsky et al., 1999; Herblot et al., 2000; Wadman et al., 1997). Ten of the 59 cases (samples 48–51, 53–57, and 59, Figure 1) did not express abnormal levels of any of the transcription factor genes described above, raising the possibility of thymocyte transformation via alternative oncogenic mechanisms.

Gene expression profiles and their biologic correlates

T cell development is a tightly regulated multistep process that involves the intrathymic differentiation, proliferation, and selection of T cell precursors (Murre, 2000; Rodewald and Fehling, 1998). Leukemic thymocytes retain many of the biologic features of normal T cell precursor subpopulations, as illustrated by shared patterns of cell surface protein expression (Reinherz and Schlossman, 1980; Bene et al., 1995). We therefore postulated that *HOX11*, *TAL1*, and *LYL1* overexpression might directly or indirectly interfere with transcriptional networks that normally regulate thymocyte proliferation, differentiation, and survival during T cell development (Look, 1997). To test this hypothesis, we used oligonucleotide microarrays (Affymetrix, HU6800) to analyze the global patterns of gene expression in 39 of the T-ALL samples with sufficient RNA for these studies (Golub et al., 1999). The microarray data are available in their entirety as Supplemental Data at http://www.genome.wi.mit.edu/mpr and http://www.cancercell.org/cgi/content/full/1/1/75/DC1.

We first asked whether the results obtained from microarray hybridizations agreed with the quantitative RT-PCR findings

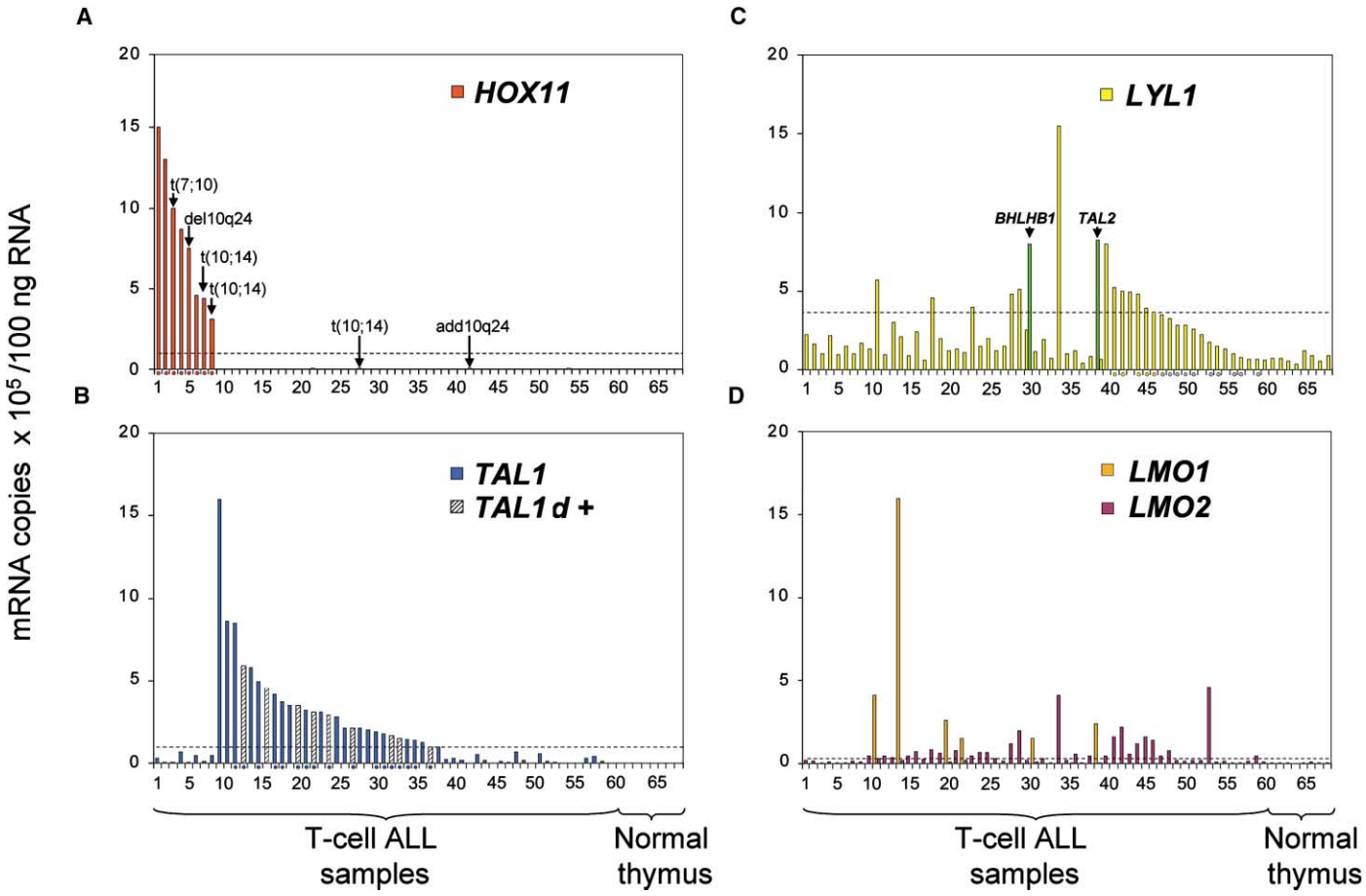


Figure 1. Quantitative RT-PCR analysis of oncogenic transcription factor genes in pediatric T-ALL samples and normal thymus controls

A: *HOX11* expression. Samples with expression levels >1 × 10^5 mRNA copies/100 ng of RNA (dotted line) were considered *HOX11*$^+$. Cases with abnormalities of chromosome band 10q24 are indicated with arrows.

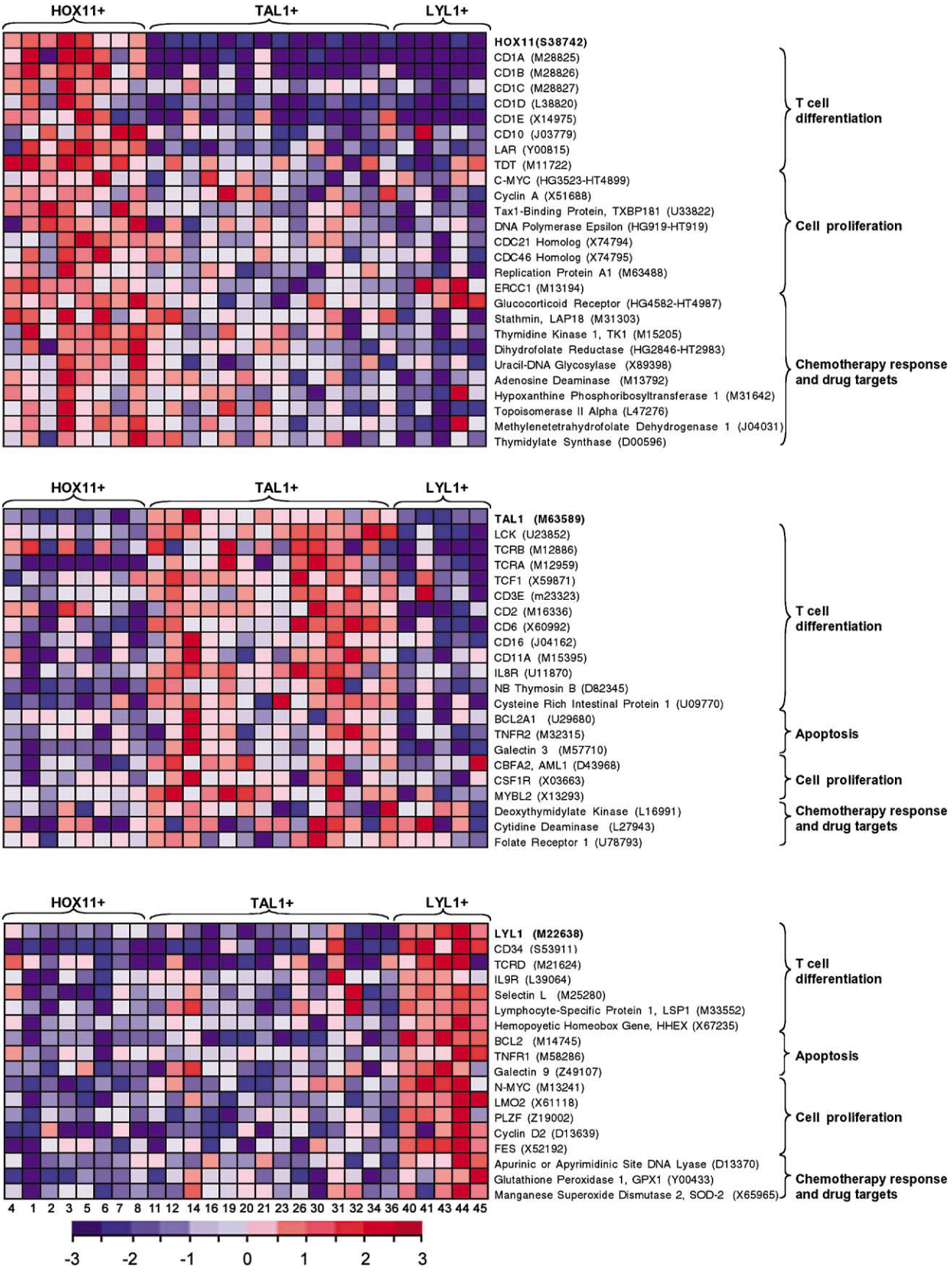
B: *TAL1* expression. Crosshatched bars indicate samples with the *Tal1d* variant band in 1p32, resulting from deletion of a 90 kb genomic DNA fragment adjacent to the *TAL1* locus. Samples showing *TAL1* expression levels above that detected in *TAL1d*$^+$ samples were considered *TAL1*$^+$ (dotted line).

C: Expression of *LYL1* (yellow bars) and other bHLH transcription factor genes (*TAL2* and *BHLHB1*) (green bars). The threshold level for *LYL1* positivity (dotted line) corresponds to five times the mean level of expression in normal thymus control samples.

D: Expression of *LMO1* (purple bars) and *LMO2* (orange bars). The threshold level for *LMO1* and *LMO2* positivity (dotted line) corresponds to five times the mean level of *LMO2* expression in normal thymus control samples. Oval symbols in panels **A**, **B**, and **C** indicate cases included in the microarray analysis.

presented in Figure 1. Normalized microarray results, plotted as increasing intensities of red (positive) or blue (negative) relative to the mean value, are shown in Figure 2. The first row of colored squares in each of the three panels depicts the expression levels of *HOX11* (top), *TAL1 (*middle), or *LYL1* (bottom) among the 27 cases independently expressing one of the three major oncogenes. The cases are arranged in the same order used to display the quantitative RT-PCR data. There was remarkable overall agreement between gene expression values obtained by these two methods.

We next surveyed genes considered to be "nearest neighbors" (Golub et al., 1999) of *HOX11*, *TAL1*, and *LYL1*, based on the close agreement of their expression profiles. Analysis of the resultant gene expression signatures (Figure 2) revealed a striking concordance with recognized stages of normal thymocyte development (Figure 3). Similar findings have been reported for B lineage tumors studied with cDNA microarray technology (Allzadeh et al., 2000). *HOX11*$^+$ cases showed increased expression of the *CD1 (A-E* family members*)*, *LAR*, and *CD10* genes in a pattern resembling that of normal cells undergoing the early cortical stage of thymocyte differentiation (Terstappen et al., 1992; Terszowski et al., 2001; Rodewald and Fehling, 1998). Many of the genes associated with *HOX11* expression are involved in cell growth and proliferation. These include adenosine deaminase (target of pentostatin, fludarabine, and 2-chlorodeoxyadenosine), DNA topoisomerase (target of the anthracyclines and epipodophyllotoxins), dihydrofolate reductase (target of methotrexate), hypoxanthine phosphoribosyltransferase 1 (modifier of the effect of antimetabolite therapy), and thymidylate synthetase (target of fluoropyrimidines and other novel folate-based inhibitors). The gene products DNA polymerase epsilon, cyclin A, Tax1 binding protein, and replication protein A1 all have prominent roles in cell proliferation. These findings are consistent with data showing that HOX11 can both immortalize hematopoietic progenitors (Keller et al., 1998) and interact directly with cell cycle regulatory proteins (Kawabe et al., 1997).


HOX11+
TAL1+
LYL1+
HOX11(S38742)
CD1A (M28825)
CD1B (M28826)
CD1C (M28827)
CD1D (L38820)
CD1E (X14975)
CD10 (J03779)
LAR (Y00815)
TDT (M11722)
C-MYC (HG3523-HT4899)
Cyclin A (X51688)
Tax1-Binding Protein, TXBP181 (U33822)
DNA Polymerase Epsilon (HG919-HT919)
CDC21 Homolog (X74794)
CDC46 Homolog (X74795)
Replication Protein A1 (M63488)
ERCC1 (M13194)
Glucocorticoid Receptor (HG4582-HT4987)
Stathmin, LAP18 (M31303)
Thymidine Kinase 1, TK1 (M15205)
Dihydrofolate Reductase (HG2846-HT2983)
Uracil-DNA Glycosylase (X89398)
Adenosine Deaminase (M13792)
Hypoxanthine Phosphoribosyltransferase 1 (M31642)
Topoisomerase II Alpha (L47276)
Methylenetetrahydrofolate Dehydrogenase 1 (J04031)
Thymidylate Synthase (D00596)
T cell differentiation
Cell proliferation
Chemotherapy response and drug targets
HOX11+
TAL1+
LYL1+
TAL1 (M63589)
LCK (U23852)
TCRB (M12886)
TCRA (M12959)
TCF1 (X59871)
CD3E (m23323)
CD2 (M16336)
CD6 (X60992)
CD16 (J04162)
CD11A (M15395)
IL8R (U11870)
NB Thymosin B (D82345)
Cysteine Rich Intestinal Protein 1 (U09770)
BCL2A1 (U29680)
TNFR2 (M32315)
Galectin 3 (M57710)
CBFA2, AML1 (D43968)
CSF1R (X03663)
MYBL2 (X13293)
Deoxythymidylate Kinase (L16991)
Cytidine Deaminase (L27943)
Folate Receptor 1 (U78793)
T cell differentiation
Apoptosis
Cell proliferation
Chemotherapy response and drug targets
HOX11+
TAL1+
LYL1+
LYL1 (M22638)
CD34 (S53911)
TCRD (M21624)
IL9R (L39064)
Selectin L (M25280)
Lymphocyte-Specific Protein 1, LSP1 (M33552)
Hemopoyetic Homeobox Gene, HHEX (X67235)
BCL2 (M14745)
TNFR1 (M58286)
Galectin 9 (Z49107)
N-MYC (M13241)
LMO2 (X61118)
PLZF (Z19002)
Cyclin D2 (D13639)
FES (X52192)
Apurinic or Apyrimidinic Site DNA Lyase (D13370)
Glutathione Peroxidase 1, GPX1 (Y00433)
Manganese Superoxide Dismutase 2, SOD-2 (X65965)
T cell differentiation
Apoptosis
Cell proliferation
Chemotherapy response and drug targets
4 1 2 3 5 6 7 8 11 12 14 16 19 20 21 23 26 30 31 32 34 36 40 41 43 44 45
-3 -2 -1 0 1 2 3

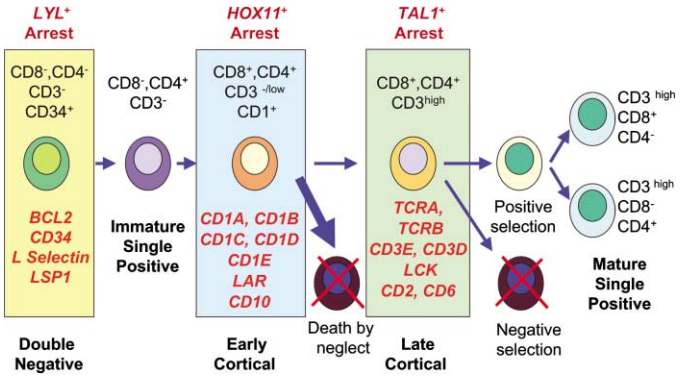


Figure 3. Correlation of gene expression profiles in *LYL1*$^+$, *HOX11*$^+$, and *TAL1*$^+$ T-ALL samples with recognized stages of thymocyte differentiation

Cell surface markers normally associated with each developmental stage (Murre, 2000) are indicated in black, with corresponding microarray findings shown in red. The most immature T cell precursors express CD34 but not CD4, CD8, or CD3. As these cells mature, they lose CD34 expression while gaining CD4 and then CD8, becoming double-positive thymocytes. Early double-positive cells initially express CD1 and CD10 (early cortical thymocytes). As they finish rearranging their T cell receptor genes, 95% of these cells fail to express a functional receptor and are ablated through a death-by-neglect mechanism. Thymocytes with functional T cell receptors gain CD3 expression (late cortical thymocytes) and undergo both positive and negative selection. Cells surviving this process proceed through a final step of differentiation in which they downregulate the expression of either CD4 or CD8 to become mature single-positive T cells.

Because the drugs used to treat human leukemias are more active in proliferating cells, these findings may explain in part the better prognosis of patients with *HOX11*$^+$ T-ALL (see Figure 5).

By contrast, the expression pattern associated with *TAL1* expression appeared to reflect the late cortical stage of thymocyte differentiation, as indicated by the upregulation of *LCK*, *TCRA*, *TCRB*, *CD2*, *CD6*, and *CD3E* (Terstappen et al., 1992; Rodewald and Fehling, 1998). High levels of *LYL1* expression were associated with an undifferentiated thymocyte phenotype characterized by increased expression of the early hematopoietic marker gene *CD34*, the cell adhesion gene L-selectin (*SELL*), the antiapoptotic gene *BCL2*, and *LSP1*, which encodes the lymphocyte-specific protein 1 (Pilarski et al., 1991; Galy et al., 1993; Ma et al., 1995; Palker et al., 1998). These results suggest that T cell oncogenes specifically interfere with transcriptional programs controlling thymocyte development, leading to stage-specific developmental arrest.

The three molecularly distinct subtypes of T-ALL also showed specific associations with known proto-oncogenes, as well as genes involved in programmed cell death (Figure 2). For example, high levels of *HOX11* correlated with increased expression of *MYC* and the proapoptotic glucocorticoid receptor gene. *TAL1* overexpression was associated with the upregulation of proto-oncogenes such as *CBFA2 (AML1)* and the *MYB*-related gene *MYBL2*, receptor genes such as *IL8R* and *CSFR1*, and the antiapoptotic gene *BCL2A1*. Finally, *LYL1* positivity was related to higher expression levels of the *MYCN*, *LMO2*, and *PLZF* proto-oncogenes, as well as the antiapoptotic gene *BCL2*. Most antineoplastic drugs are thought to act through the mitochondrial apoptotic machinery, and their cytostatic effects are inhibited by BCL2 and its related prosurvival family members (Reed, 1995). Thus, the upregulation of *BCL2* and *BCL2A1* in *LYL1*- and *TAL1*-overexpressing cases may explain their relative resistance to chemotherapy (see Figure 5), while the exquisite responsiveness of *HOX11*$^+$ cases could partly reflect the downregulation of survival factors in early cortical stage thymocytes, most of which are targeted for "death by neglect."

Hierarchical clustering of T-ALL cases based on gene expression patterns

Although helping to identify the sets of genes coordinately expressed with *HOX11*, *TAL1*, and *LYL1*, the nearest neighbor analysis depicted in Figure 2 provided little useful information about the 10 cases that lacked discernible expression of these oncogenes (designated Other in Figure 4) or the two additional cases expressing both *LYL1* and *TAL1* (Mixed). To gain insight in the molecular characteristics of these poorly understood cases, we generated hierarchical clusters based on the 72 genes whose expression patterns best distinguished between each group of *HOX11*$^+$, *TAL1*$^+$, *LYL1*$^+$, and other cases in pairwise comparisons (as defined by the permutation distribution of the maximum t statistic, $P < 0.30$).

As shown in Figure 4, the *HOX11*$^+$, *TAL1*$^+$, and *LYL*$^+$ samples detected by RT-PCR are grouped together within major branches of the dendrogram. The branch containing the *HOX11* samples (H) comprises two subgroups, one containing most of the *HOX11* RT-PCR-positive cases (H1) and the other consisting primarily of *HOX11*-like samples that lacked *HOX11* expression by RT-PCR (H2). Surface immunophenotyping indicated that these subgroups had related but distinct immunophenotypes (Table 1). True *HOX11* samples were primarily CD1$^+$, CD10$^{+/-}$, CD4$^+$, CD8$^+$, and CD3$^-$ (early cortical thymocytes), while the *HOX11*-like samples were primarily CD1$^{+/-}$, CD10$^+$, CD4$^+$, CD8$^+$, and CD3$^+$ (early cortical thymocytes with acquired CD3 surface expression). Similarly, the central cluster of *TAL1*$^+$ samples contained two subgroups, a larger one comprising most of the true *TAL1*$^+$ samples (T1) and a second consisting mainly of *TAL1*-like samples (T2). A third, smaller branch (M) emerged from the hierarchical analysis, and was characterized by a global pattern of increased expression of many of the genes that distin-

Figure 2. *HOX11*$^+$, *TAL1*$^+$, and *LYL1*$^+$ nearest neighbor analysis

Each row of squares shows the expression pattern of a particular gene selected by nearest neighbor analysis (Golub et al., 1999), while each column represents 1 of the 27 samples positive for *HOX11*, *TAL1*, or *LYL1* by RT-PCR (see Figure 1). The genes depicted were chosen from the top 200 nearest neighbors of each major oncogene (boldface type) on the basis of their potential functional relevance and then were grouped according to their involvement in T cell differentiation, apoptosis, cell proliferation, or chemotherapy response. Expression levels for each gene were normalized across the samples; levels greater than or less than the mean (by as much as three standard deviations) are shown in shades of red or blue, respectively. Numbers at the bottom correspond to the numbers of the samples in Figure 1. For a complete list of gene names, accession numbers, and raw expression values, see Supplemental Data at http://www.genome.wi.mit.edu/mpr and http://www.cancercell.org/cgi/content/full/1/1/75/DC1.

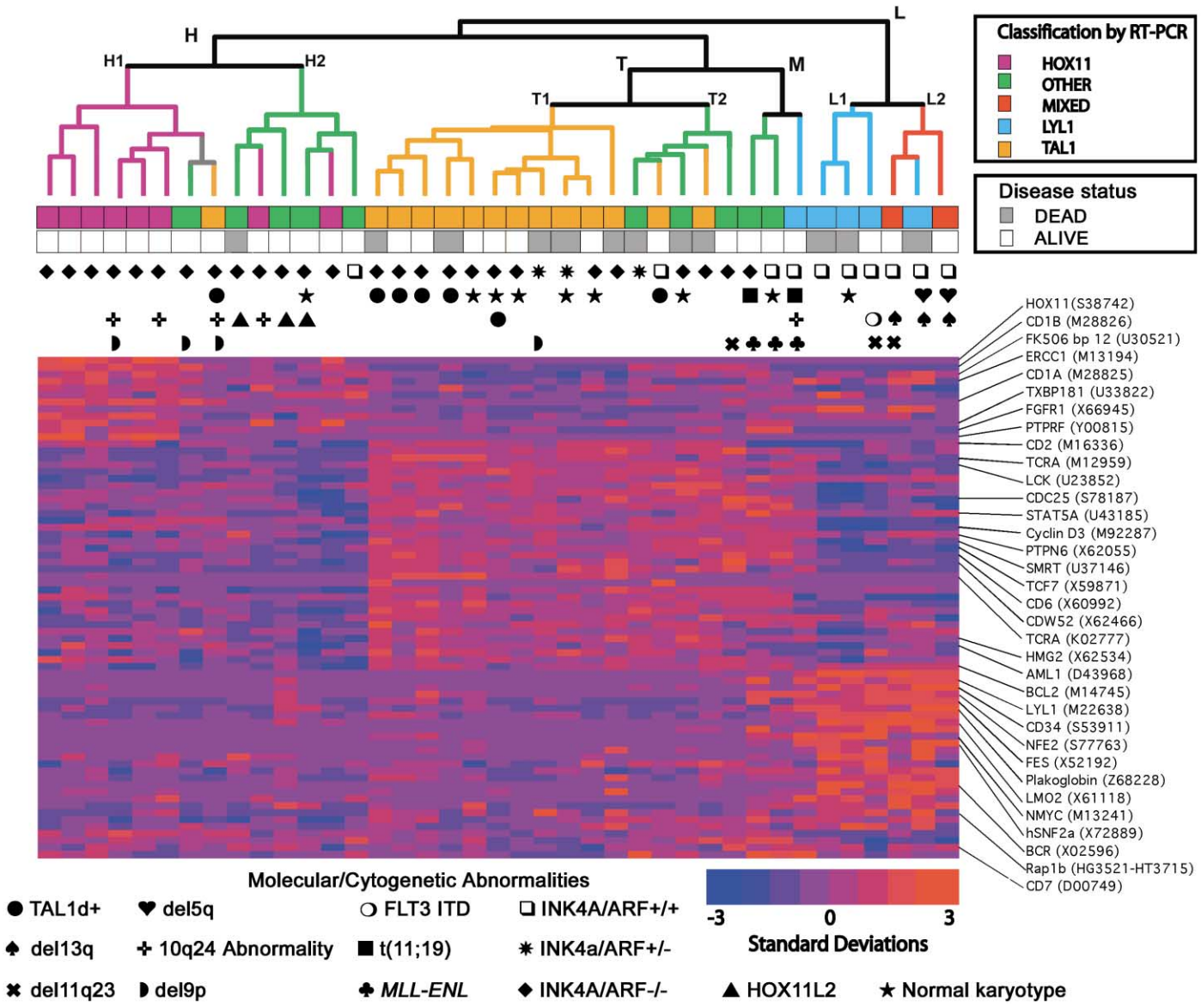


Figure 4. Hierarchical cluster analysis of gene expression data

Expression profiles of 72 genes were selected by permutation test analysis as those best distinguishing among 39 *HOX11*$^+$, *TAL1*$^+$, *LYL1*$^+$ cases and unclassified samples (Other). The dendrogram (top) shows the relatedness of gene expression among samples and is color coded according to the quantitative RT-PCR category of each sample (see Figure 1). Clinical outcome data are reported as horizontal bars with open boxes representing survivors and dark boxes deceased patients. Cytogenetic and molecular abnormalities are indicated by discrete symbols defined at the bottom of the figure. Each column represents a T-ALL mRNA sample and each row a gene on the microarray. Genes are grouped into four consecutive categories: higher in *HOX11*$^+$ than in *TAL1*$^+$, *LYL1*$^+$, or Others; higher in *TAL1*$^+$ than in *HOX11*$^+$, *LYL1*$^+$, or Others; higher in *LYL1*$^+$ than in *HOX11*$^+$, *TAL1*$^+$, or Others; and finally higher in Others than in *HOX11*$^+$, *TAL1*$^+$, or *LYL1*$^+$; and are listed within each category in order from lowest to highest P value. A complete list of genes and P values is available as Supplemental Data at http://www.genome.wi.mit.edu/mpr and http://www.cancercell.org/cgi/content/full/1/1/75/DC1. Gene expression values are normalized and color coded, as indicated by the scale beneath the graph. Major branches in the dendrogram are designated by the first letter of the dominant oncogene (e.g., H, H1, H2, for *HOX11*).

guished among the other three groups. Interestingly, two of these three cases had the t(11;19)(q23;p13.3), which produces the *MLL-ENL* fusion gene (Rubnitz et al., 1999a), while the remaining case had a normal karyotype. *MLL-ENL* RT-PCR analysis, performed in 59 samples**,** revealed the *MLL-ENL* fusion transcript in only three cases, all in the M cluster, including the case with a normal karyotype. This result illustrates the power of DNA microarray analysis to group samples according to specific mechanisms of leukemic transformation.

Finally, the *LYL*$^+$ cluster (L) included two branches. One contained three of the true *LYL*$^+$ samples (L1), including the only T cell sample in this series with the *FLT-3* internal tandem duplication, which is often identified in acute myeloid leukemias (Nakao et al., 1996; Yokota et al., 1997). These leukemias also expressed high levels of CD34 as well as myeloid markers, consistent with differentiation arrest in the early stages of T cell development, when T progenitor cells are migrating from the bone marrow to the thymus. A second branch (L2) contained

Table 1. Cell surface antigen expression among T-ALL samples

	CD34$^+$	CD10$^+$	CD1$^+$	DP[a] CD4$^+$ CD8$^+$	DN[b] CD4$^-$ CD8$^-$	CD3$^+$	MY[c] CD13$^+$/33$^+$
Cluster H n = 14	43	78	75	78	0	36	7
HOX11$^+$ In cluster H n = 8	37	62	100	62	0	0	0
HOX11$^-$ In cluster H n = 6	50	100	50	100	0	83	16
Cluster T n = 14	71	27	46	73	13	66	0
Cluster T1 n = 9	78	20	40	70	20	90	0
Cluster T2 n = 5	60	40	60	80	0	20	0
Cluster L n = 6	100	33	0	0	83	33	100

The values are percentages of positive samples.
[a] Double-positive thymocytes.
[b] Double-negative thymocytes.
[c] Myeloid lineage.

a *LYL*$^+$ sample and two samples with simultaneous expression of *TAL1* and *LYL1* by quantitative RT-PCR. Indications of multistep mutational pathways emerged when samples were analyzed by quantitative DNA PCR for deletions of *P16/INK4A-P14/ARF* (Drexler, 1998; Okuda et al., 1995). In our series of T-ALL cases, homozygous deletions of this gene were found in most samples in clusters H and T (Figure 4), which included *HOX11*$^+$ and *TAL1*$^+$ cases as well as cases with similar overall patterns of gene expression. Homozygous deletion of *P16/INK4A-P14/ARF* was not detected in 2 of the three *MLL-ENL*$^+$ cases in the M cluster, nor in any of the cases grouped into cluster L, which comprise *LYL1*$^+$ cases as well as mixed cases expressing both *TAL1* and *LYL1* (Figure 4). The exclusive presence of cytogenetic features such as the 5q- and 13q-deletions within the L2 subgroup (Figure 4) further attests to the ability of our hierarchical clustering approach to group samples with common mechanisms of transformation and suggests that tumor suppressor genes in the 5q and 13q regions are inactivated as part of a distinct oncogenic pathway that gives rise to these leukemias.

Oncogene discovery through microarray expression analysis: *HOX11L2* is activated in T-ALL samples

The observation that T-ALL leukemia cases with *MLL-ENL* rearrangement and recurrent cytogenetic abnormalities were grouped together in our hierarchical clustering analysis illustrates the ability of gene expression profiling to identify cases with common mechanisms of transformation. Results of this analysis also suggested that cases without defined oncogene activation that clustered with the *HOX11*, *TAL1*, or *LYL1* samples likely harbor related but as yet unidentified oncogenes. To test this hypothesis, we used quantitative RT-PCR to analyze the expression of *HOX11L1* and *HOX11L2*, two homeobox genes that are not included in the Affymetrix 6800 microarray but are functionally and structurally related to *HOX11*. *HOX11L1* was expressed at comparable low levels in both normal thymus and T-ALL samples, indicating that it was not overexpressed in this group of leukemias. *HOX11L2*, on the other hand, was expressed at high levels ($>10^6$ copies per 100 ng of RNA) in six of the T-ALL samples (29, 48, 49, 51, 54, and 55, as numbered in Figure 1), but was undetectable in normal thymus and the other T-ALL samples. Three of the six *HOX11L2*$^+$ cases (closed triangles in Figure 4) had sufficient RNA for microarray analysis. Their location in the *HOX11*-related "H2 cluster" of the hierarchical dendrogram confirms our hypothesis that cases with gene expression signatures resembling *HOX11*$^+$ cases might be transformed through the effects of highly related oncogenes operating through similar oncogenic pathways.

Statistical analysis to identify the genes that were differentially expressed in *HOX11*$^+$ versus *HOX11L2*$^+$ cases revealed increased expression (Permax P value < 0.30, see Experimental Procedures) of *HOX11* itself and eight additional genes in *HOX11*$^+$ cases (Fuse binding protein 2 [*FBP2*; U69126], DXS9879E [*ITBA2*; X92896], H2AZ histone [*H2AZ*; M37583], glycerladehyde-3-phosphate dehydrogenase [*G3PD*, EC 1.2.1.12; X01677], SW1/SNF complex 155 kDa subunit [*BAF155*; U66615], FYN binding protein [*FYB*; U93049], proteasome subunit α 1 [*PSMA1*; M64992] and tubulin β 5 [V00599]). None of the genes on the microarray were expressed at significantly higher levels in *HOX11L2*$^+$ cases. Thus, despite the marked similarities in gene expression profiles between *HOX11*$^+$ and *HOX11L2*$^+$ cases, *HOX11*$^+$ cases are distinguished by increased expression of genes involved in signal transduction and the chromatin-mediated control of gene expression (see Discussion).

Oncogene activation and gene expression signatures have prognostic relevance

To assess the prognostic significance of these findings, we first analyzed the survival of 58 eligible patients from the 59 whose leukemic cells were analyzed for *HOX11*, *HOX11L2*, *TAL1*, or *LYL1* expression by quantitative RT-PCR. Preliminary comparison of the Kaplan-Meier plots showed no significant difference between the *TAL1*$^+$ and *LYL1*$^+$ groups, prompting us to com-

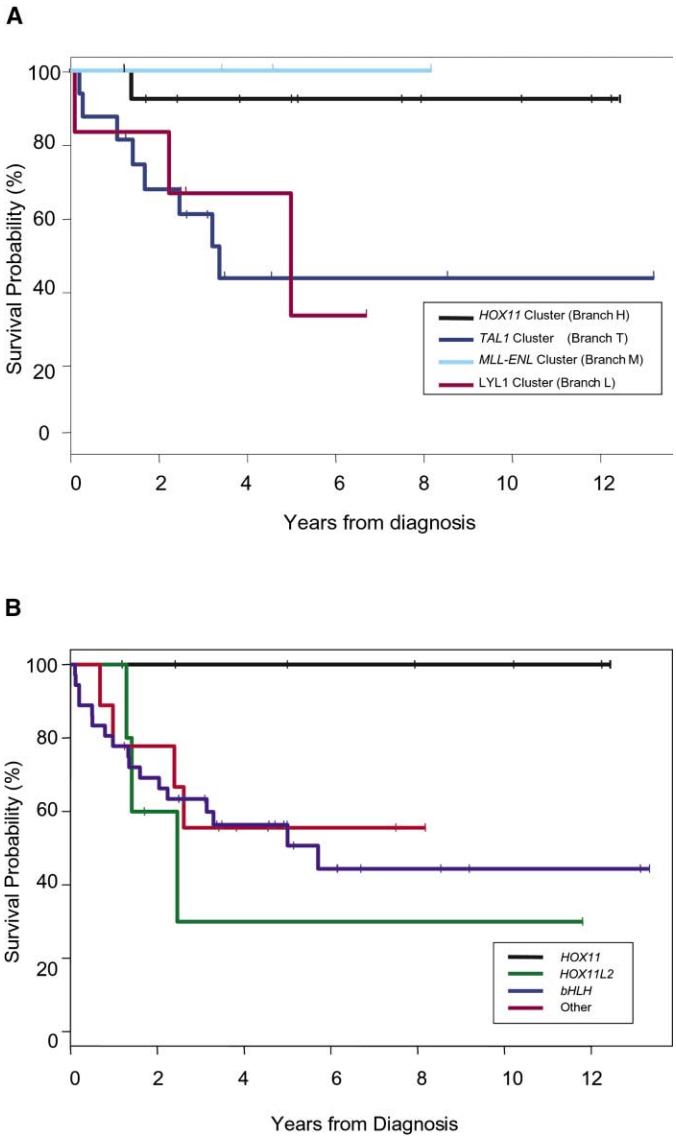


Figure 5. Clinically important T-ALL subgroups identified by gene expression profiling

A: Kaplan-Meier plots of overall survival among patients with a *HOX11*$^+$, *HOX11L2*$^+$, *bHLH*$^+$, or unclassified (Other) gene expression signature by RT-PCR analysis (*TAL1*$^+$, *TAL2*$^+$, *bHLHB1*$^+$, and *LYL1*$^+$ samples combined as bHLH$^+$).

B: Kaplan-Meier plots of overall survival for subgroups recognized by hierarchical clustering of DNA microarray data, which subdivided the samples into four main clusters (H, T, M, and L; see Figure 4). Tick marks on the curves represent surviving patients.

bine these two cohorts (bHLH$^+$) for further analysis. As shown in Figure 5A, constitutive expression of *HOX11* was associated with a favorable prognosis: probability of survival at 5 years was 100% ± 0% standard error (SE), compared with 30% ± 24% for the *HOX11L2* group and 51% ± 9% for the bHLH$^+$ group ($P = 0.02$ by log-rank analysis, comparing *HOX11*$^+$ with all other patients). Patients whose leukemic cells lacked expression of *HOX11*, *HOX11L2*, or *bHLH* oncogenes had essentially the same probability of 5 year survival as the latter two groups ($P = 0.81$). Primary drug resistance, demonstrated by the failure to achieve complete remission, was noted only in patients with overexpression of *TAL1* or *LYL1*.

Similar results were obtained when the Kaplan-Meier analysis focused on the major groups defined by hierarchical clustering of gene expression signatures (Figure 5B): probability of survival at 5 years was 92% ± 8% for the *HOX11*$^+$ cluster versus 43% ± 19% and 33% ± 19% for the *TAL1*$^+$ and *LYL1*$^+$ clusters ($P = 0.03$). None of the three patients in the small cluster containing the *MLL-ENL* cases have died. Although based on a small number of samples, this result agrees with a previous report suggesting that *MLL-ENL* translocations in T-ALL may not carry the dire prognosis associated with related translocations in infants and older children with an early B lineage ALL immunophenotype (Behm et al., 1996; Rubnitz et al., 1999b).

Discussion

The dramatically different clinical courses of T-ALL patients treated with the same intensive multidrug regimens support the interpretation that ALL arising in thymic lymphocytes comprises several biologically distinct diseases. This variability likely reflects a molecular heterogeneity that has not been appreciated from characterization of leukemic T cells by conventional methods, as recently demonstrated in a microarray study for diffuse large B cell lymphoma (Allzadeh et al., 2000). Thus, gene expression analysis using oligonucleotide or cDNA microarrays offers a novel tool for delineating molecular pathways that drive the malignant transformation of developing thymocytes.

The results reported here identify previously unrecognized molecular subtypes of T-ALL and link the activation of particular oncogenes to defined stages of normal thymocyte development. Highly favorable clinical outcomes were observed for patients in the *HOX11*$^+$ cluster, whose cell samples showed a pattern of gene expression resembling that of early cortical thymocytes. The better therapeutic responsiveness of this subgroup may be explained by several distinctive features of *HOX11*$^+$ lymphoblasts, including the expression of genes associated with increased cell proliferative activity and the lack of expression of *BCL2* and related antiapoptotic genes. Apoptosis is a major regulatory mechanism during normal T cell development, eliminating the more than 90% of normal cortical thymocytes unable to express functional T cell receptors (Vacchio et al., 1998). Thus, *HOX11*$^+$ lymphoblasts appear to be arrested at a stage of thymocyte development that is especially responsive to drug-induced programmed cell death.

Less favorable outcomes were observed in subgroups defined by gene expression profiles characteristic of *TAL1*$^+$ or *LYL1*$^+$ samples, which resemble late cortical and early pro-T thymocytes, respectively. Drug resistance in *LYL*$^+$ samples may be explained by the fact that early double-negative pro-T cells express high levels of *BCL2* and show increased resistance to apoptosis (Veis et al., 1993). *TAL1*$^+$ cells appear to upregulate *BCL2A1* (also known as *BFL1*) and other antiapoptotic molecules normally induced by signaling through the TCR in late cortical thymocytes (Tomayko et al., 1999), suggesting different mechanisms of treatment resistance in *TAL1*$^+$ and *LYL1*$^+$ cases. We find it surprising that *LYL1* and *TAL1* overexpression is associated with maturational arrest at opposite ends of the thymocyte developmental spectrum, despite structural and biochemical data suggesting that these two proteins might act quite similarly as inhibitors of normal E12/E47 bHLH activity

(Murre, 2000; Baer, 1993; Miyamoto et al., 1996). This may reflect differences in the stages of thymocyte differentiation at which these oncogenes are activated. Alternatively, the key transformation event involving bHLH oncogenes may occur early in the $CD4^-/CD8^-$ cell stage. In this model, TAL1 may abrogate the normal E2A-induced arrest of further differentiation (Engel et al., 2001) more effectively than LYL1, leading to leukemias that resemble more mature $CD4^+/CD8^+$ double-positive thymocytes.

Our microarray studies of leukemic thymocytes revealed distinctive gene expression signatures that are strongly associated with specific oncogenic transcription factors. In some instances, closely related signatures were found in samples lacking activation of known T-ALL oncogenes, leading us to predict alternative oncogenic transcription factors that could initiate similar patterns of gene expression. In experiments based on this hypothesis, we identified *HOX11L2* overexpression as an oncogenic event in *HOX11*-negative samples that exhibited the gene expression signature associated with bona fide *HOX11*-expressing cases. *HOX11L2* is an orphan homeobox factor very similar to *HOX11*, and has been shown to be essential for the normal development of the ventral medullary respiratory center, in that its deficiency in mice leads to a respiratory failure resembling congenital central hypoventilation syndrome in humans (Shirasawa et al., 2000). The recent report by Bernard et al. (2001) of a novel cryptic recurrent translocation t(5;14)(q35;q32) in T-ALL resulting in aberrant *HOX11L2* expression reinforces the role of this homeobox factor as a T-ALL oncogene.

Given the marked similarity in gene expression profiles between $HOX11^+$ and $HOX11L2^+$ cases, it is surprising that these two groups of patients have such different treatment outcomes, and additional patients will need to be studied to confirm this result. Of the eight genes that were more highly expressed by $HOX11^+$ leukemias, *FBP2*, *BAF155*, and *FYB* encode regulatory proteins that might provide insight into the dissimilar clinical responses. The far upstream binding protein 2 (FBP2) regulates alternative mRNA splicing through binding to intronic splicing enhancer sequences (Min et al., 1997), while BRG1-associated factor 155 (BAF155) is the human homolog of the yeast protein SWI3, a component of the SWI/SNF complex that regulates gene expression through chromatin remodeling. Two components of the corresponding mammalian complex, BAF47/SNF5 (Roberts et al., 2000; Versteege et al., 1998) and BRG1 (Wong et al., 2000), are known to be tumor suppressors, and BAF155 together with BRG1 has been shown to interact with cyclin E, with BRG1 specifically causing cell growth arrest (Shanahan et al., 1999). FYN binding protein (FYB) is an important positive regulator of T cell activation and couples TCR signals to integrin activation and adhesion (Geng et al., 2001; Griffiths et al., 2001; Peterson et al., 2001).

In contrast to the inclusive microarray analysis employed by Allzadeh and coworkers to characterize subgroups of B cell lymphoma (Allzadeh et al., 2000), we chose to focus genes that best distinguish among cases expressing known T-All oncogenes. This approach was based on the hypothesis that dominant oncogenic transcription factors in this disease, such as *HOX11*, *TAL1*, and *LYL1*, stand at the top of regulatory cascades whose aberrant activation can lead to T cell neoplasia. Our "hypothesis driven" approach to hierarchical clustering has enabled us to integrate complex gene expression patterns into a conceptual framework with biologic relevance to T cell ALL. We would stress, however, that only a small subset of the genes comprising each gene expression signature are likely to be directly regulated by the oncogenic transcription factors themselves. Since many of the specifically expressed genes appear to reflect a specific stage of T cell developmental arrest, it will be important to compare gene expression profiles in leukemic T lymphoblasts versus subsets of normal thymocytes at different stages of differentiation to identify transcriptional programs that are directly linked to leukemic transformation.

Our studies indicate that wider application of gene expression profiling in T-All would help to identify therapeutically relevant diagnostic subgroups. It may also be possible, given sufficient numbers of patients, to identify signal transduction pathways that are vital to the proliferation and survival of individual subgroups, making proteins within these pathways attractive targets for new therapeutic approaches.

Experimental procedures

Patient material

Samples of cryopreserved lymphoblasts from 59 children and young adults with T-ALL, treated in Total Therapy studies XI–XIII at St. Jude Children's Research Hospital (TN), were obtained with informed consent at the time of diagnosis, before any chemotherapy was given. The median age of the patients was 9.3 years (range 0.5–18.8), the male to female ratio was 3.0, and the leukocyte count at diagnosis was 2,300–917,000 per mm^3 (median, 164,000). Mean lymphoblast percentage in the samples analyzed was 91% ± 10% SD. Six patients had CNS disease at presentation, and mediastinal masses were present in 36. One case with less than one year of followup was excluded from survival analysis. Lymphoid cells were also obtained (with informed consent) from normal thymic tissue removed at the time of cardiac surgery.

DNA and RNA preparation

RNA was prepared from cryopreserved lymphoblasts with RNAqueous reagents (Ambion) according to the manufacturer's instructions and quantitated spectrophotometrically. The quality of the purified RNA was assessed by visualization of 18S and 28S RNA bands under ultraviolet light after electrophoresis through denaturing agarose gels and staining with ethidium bromide. Genomic DNA from each sample was extracted with a commercial kit (GENTRA) following the manufacturer's instructions, spectrophotometrically quantified, and stored at −20°C until analysis.

Primers and probes

Primers and probes were designed with the assistance of the computer program Primer Express (Perkin-Elmer Applied Biosystems) and with flanking intron-exon boundaries to prevent amplification from any residual genomic DNA, while avoiding areas involved in the generation of alternative spliced mRNAs. In the case of *TAL2*, *BHLHB1*, *HOX11L1*, and *HOX11L2*, which lacked suitable intron-exon boundaries for primer-probe design, the amount of residual genomic DNA in each sample was determined by simultaneous quantitation of these genes on RNA specimens in the presence and absence of reverse transcriptase. *GAPDH* FW: 5′-GAAGGTGAAGGTCGGAGT-3′, *GAPDH* RV: 5′-GAAGATGGTGATGGGATTTC-3′, *GAPDG* Probe: 5′-VIC-CAAGCTTCCCGTTCTCAGCC-TAMRA-3′, *TAL1* FW: 5′-GAAGAGGAGA CCTTCCCCCT-3′, *TAL1* RV: 5′-GGTGAAGATACGCCGCACA-3′, *TAL1* Probe: 5′-FAM-TGAGATGGAGATTACTGATGGTCCCCA-TAMRA-3′, *TAL2* FW: 5′-GCCTGCAACAAACGGGAGT-3′, *TAL2* RV: 5′-AGAGTTCTGTCCTC CAGGCCT-3′, *TAL2* Probe: 5′-FAM-CTCTTCCCTCAAGGACCCCACCTGC-TAMRA-3′, *LYL1* FW: 5′-CCCACTTTGGCCCTGCA-3′, *LYL1* RV: 5′-GGTCCTGCTGGCCCAATGT3′, *LYL1* Probe: 5′-FAM-TACCACCCTCACC CCTTCCTCAACAGTGTC-TAMRA-3′ *BHLHB1* FW: 5′-GGCAGTGGCTT CAAGTCGTC-3′, *BHLHB1* RV: 5′-TCCGGCTCTGTCATTTGCTT-3′, *BHLHB1* Probe: 5′-FAM-TCGTCCAGCACCTCGTCGTCTACG-TAMRA-3′, *HOX11* FW: 5′-TGGATGGAGAGTAACCGCAGAT-3′, *HOX11* RV: 5′-GGGCGTCCGGTTCTGATA-3′, *HOX11* Probe: 5′-FAM-CACAAAGGACAG GTTCACAGGTCACCC-TAMRA-3′, *HOX11L1* FW: 5′-GGATGCTGGGTC CACACAAC-3′, *HOX11L1* RV: 5′-CAGGATCTGATCGATGCCGA-3′,

HOX11L1 Probe: 5′-FAM-TCCCACACCACGAGCCAATCAGC-TAMRA-3′, *HOX11L2* FW: 5′-GCCCAAGCGTAAGAAGCCGC-3′, *HOX11L2* RV: 5′-AGC GCTTTTCCAGCTCGCAG-3′, *HOX11L2* Probe: 5′-FAM-CACGTCCTTTTC CCGGGTGCAGA-TAMRA-3′, *LMO1* FW: 5′-TCTACACCAAGGCCAACC TCA-3′, *LMO1* Probe: 5′-FAM-CGCGACTACCTGAGGCTCTTTGGCA-TAMRA-3′, *LMO1* RV:5′-TGCAAGCAGCACAGTTCCC-3′, *LMO2* FW: 5′-TACAAACTGGGCCGGAAGC-3′, *LMO2* Probe: 5′-FAM-CGGAGAGACTAT CTCAGGCTTTTTGGGC-TAMRA-3′, *LMO2* RV:5′-CTTGTCACAGGATGCG CAGA-3′. Unmodified primers and 5′-FAM, 3′-TAMRA or 5′-HEX, 3′-TAMRA labeled probes were synthesized by Integrated DNA Technologies, while GAPDH 5′-VIC, 3′-TAMRA labeled probe was synthesized by PE Applied Biosystems. Tal1d, FLT3 ITD analysis by PCR and *MLL-ENL* RT-PCR fusion transcript detection were performed as previously described (Meshinchi et al., 2001; Pongers-Willemse et al., 1999; Rubnitz et al., 1996).

Real-time RT-PCR

Real-time quantitative RT-PCR analyses were performed with an ABI PRISM 7700 Sequence Detection System instrument (Perkin-Elmer Applied Biosystems) in a total volume of 50 μl containing 1× Taq DNA polymerase buffer (Perkin-Elmer Applied Biosystems) supplemented with 1 μM 5-carboxy-X-Rhodamine (5-ROX) (Molecular Probes), 200 μM dATP, dCTP, dGTP, and dTTP (Roche), 100–300 nM of each primer, 150 nM of the taqman probe, 10 units of RNase inhibitor (Perkin-Elmer Applied Biosystems), 5 units of reverse transcriptase (Perkin-Elmer Applied Biosystems), 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), and 1.5–2.5 mM $MgCl_2$. One-step RT-PCR consisted of an initial incubation at 48°C for 30 min, followed by a denaturation step at 95°C for 10 min, and amplification for 40 cycles of 15 s at 95°C and 1 min at the optimized annealing temperature. 100 ng of total RNA was analyzed for each case. RNA samples were treated before analysis with DNase I in order to eliminate residual genomic DNA. Each experiment included three nontemplate controls to detect any template contamination; a control lacking reverse transcriptase was included for each sample to detect any residual genomic DNA. Standard curves were constructed by serial 10-fold dilutions from 10^8 to 10^2 copies of in vitro-transcribed RNA of each gene to be quantified. The reproducibility of the quantitative measurements was evaluated by conducting triplicate PCR assessments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was analyzed for each sample in parallel wells to assess the integrity of the RNA. *INK4A-ARF* deletion status was determined by multiplex real-time quantitative PCR of *INK4A/ARF* and β-actin as described previously (Carter et al., 2001).

Microarray expression analysis

A total of 10 μg of total RNA from each sample was used to prepare biotinilated target RNA as previously described with minor modifications (Golub et al., 1999; Tamayo et al., 1999; Wodicka et al., 1997). A complete description of biochemical and analytical procedures is available as Supplemental Data at http://www.genome.wi.mit.edu/mpr and http://www.cancercell.org/cgi/content/full/1/1/75/DC1. To identify the genes whose expression patterns best distinguished among HOX11+, TAL1+, LYL1+, and other cases, we analyzed the permutation distribution of the maximum t statistic using the Permax program (biowww.dfci.harvard.edu/gray/permax.html). Affymetrix average difference values were transformed to base two logarithms after raw values under 100 were raised to this value. 72 genes with a P value of <0.30 by permutation test analysis were used to build a hierarchical tree (http://www.insightful.com/products/splus). We chose a cutoff Permax P value of <0.30, even though P values <0.05 are typically regarded as statistically significant. In our analysis, the Permax P value is calculated by comparing observed t statistics to the permutation distribution of the largest t statistic obtained for the 5990 genes. Thus, a Permax P value of 0.30 corresponds approximately to a t statistic of 4.73 with 17 degrees of freedom, which has a nominal P value of 0.000097.

To test whether the structure of the dendrogram shown in Figure 4 might be highly dependent on the limited number of genes used or our method of gene selection, we increased the number to 116 genes selected based on a P value <0.5 instead of <0.3, and obtained a nearly identical dendrogram.

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