Gene Expression Profiles in a Panel of Childhood Leukemia Cell Lines Mirror Critical Features of the Disease

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Abstract

The development of new drugs against cancer requires established cell lines. They are needed for *in vitro* studies to identify candidate drugs and in xenograft models to measure drug efficacy *in vivo*. Specific criteria need to be fulfilled by cell lines used in the evaluation of potential novel therapeutic agents. It is imperative that they display the features of the particular cancer under investigation. Given the documented heterogeneity of cancers, relevant subtypes need to be represented. In this study, we have examined these aspects for pediatric acute lymphoblastic leukemia. A panel of 13 leukemia cell lines recently established in our laboratory was analyzed. We used cDNA microarrays to define the gene expression profiles and compared the data with immunophenotyping and cytogenetic analyses. The expression profiles obtained showed excellent concordance with corresponding protein levels. Importantly, the panel of lines displayed the critical genetic features identified in clinically important acute lymphoblastic leukemia subtypes in childhood leukemia patients.

Introduction

Cancer therapy has optimal chance of success if tailored to the exact tumor type of the patient. High specificity can be achieved by novel therapeutics designed for particular molecular alterations in cancer cells. Herceptin and Gleevec are examples of this new class of drugs. Heterogeneity is the hallmark of cancers, most prominently demonstrated by GEPs.[2] The microarray technique allows molecular classification of cancers, according to their expression profiles, as evidenced by the seminal work on lymphoid tumors (1, 2). Moreover, these studies showed that the use of DNA microarrays provides a strategy for discovering and predicting "cancer classes" or subtypes of cancer, independent of previous knowledge of the biology of the cancers. This in turn will improve the risk stratification of patients and hence the precision and power of clinical trials. In addition, the GEPs provide information on potential new targets for therapy, providing the basis for novel, target-directed therapies.

The evaluation of potential drug leads requires a series of investigations, and they invariably involve the use of established cell lines. Depending on the design of the study, large panels or specifically designed compounds are tested for their growth inhibitory capacity, often in parallel with more specific analyses, *e.g.,* to assess induction of apoptosis. The National Cancer Institute's Developmental Therapeutics Program has carried out intensive studies of 60 cancer cell lines (the NCI60) derived from tumors from a variety of tissues (3, 4). The insight gained from GEPs has led to novel high-throughput approaches where drug leads are assessed for their effects on the expression of sets of genes, prominently expressed in particular cancers. Successful *in vitro* tests are then validated by *in vivo* studies, often using xenograft models where the reduction of tumor burden is measured in immunodeficient animals, again making use of established cell lines. Importantly, any cell line used in such drug evaluation studies needs to fulfill particular criteria. It is imperative that cell lines display the features of the particular cancer under investigation. Given the documented heterogeneity of cancers, relevant subtypes need to be represented. In this study, we have addressed these issues in respect to pediatric ALL.

Pediatric ALL is one of the success stories of cancer therapy, and long-term survival has reached 70–75% (5–9). These survival rates demonstrate that well-designed large clinical trials have been very effective. Current treatment protocols make use of risk-directed therapy with the aim of administering intensified therapy to those patients with a high likelihood of relapse and decreasing toxicity for those with a lower risk of relapse, while maintaining high cure rates. The stratification of patients is based on clinical and laboratory features at diagnosis. However, the clinician treating pediatric ALL patients is faced with a major problem. Substantial numbers of patients currently classified and treated as standard risk patients continue to relapse (5, 7, 10–12). The heterogeneity among pediatric ALL has been addressed by GEP studies of large patient cohorts (13–15), and the data obtained will form the basis for the identification of therapies tailored to relevant subgroups. To determine whether cell lines established from patients with pediatric ALL would be suitable for the evaluation of novel drugs, we investigated whether they mirror the critical features characteristic for

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[2] The abbreviations used are: GEP, gene expression profile; ALL, acute lymphoblastic leukemia; T-ALL, acute lymphoblastic leukemia of T-cell phenotype.

Table 1 Characterization of childhood ALL cell lines

Cell line	Origin[a]	Immunophenotype[b]								Reference
		CD2	CD3	CD4	CD5	CD7	CD10	CD19	MHCII	
T-ALL										
PER-117	R	–[c]	–	–	–	28	–	–	–	16
PER-255	D	99	–	99	100	73	–	–	–	17, 18
PER-427	D	92	98	100	61	89	–	–	45	
PER-487	D	50	47	–	51	92	–	–	–	
PER-537	D	67	40	–	28	27	–	–	–	
PER-550	R	88	47	30	37	36	–	–	–	
pre-B ALL										
PER-145	R3	–	–	–	26	–	70	ND	56	19, 20
PER-278	D	–	–	–	42	–	22	75	74	21
PER-371	D	–	–	–	–	–	76	84	74	
B-ALL										
PER-377	R	–	–	–	–	14	–	95	80	22, 23
PER-495	R	–	–	ND	–	–	8	16	16	
Infant leukemia										
PER-485	R	–	–	98	98	92	–	–	ND	
PER-490	D	–	–	–	–	24	–	93	ND	

[a] Cell line was established from D (diagnosis), R (relapse), or R3 (third relapse).
[b] Percentage of cells staining as determined by immunofluorescence.
[c] –, <3% of cells staining; ND, not determined.

subgroups of patients. In this study, we combined cytogenetic and immunophenotype analysis with GEPs to analyze a panel of pediatric leukemia cell lines established in our laboratory.

Materials and Methods

Patient Samples and Cell Lines. Bone marrow specimens were obtained from pediatric leukemia patients diagnosed and treated at the Princess Margaret Hospital for Children (Perth, Australia). Informed consent was obtained from parents, patients, or both, as deemed appropriate. The cell lines were generated according to our method published previously (16), and some of the lines were reported previously (16–23). Two of the cell lines of T-ALL, PER-427 and PER-487, required the addition of 300 units/ml interleukin-2 to the growth medium. Seven cell lines were derived from bone marrow specimens obtained at first diagnosis and six from relapse specimens, as indicated in Table 1. Immunophenotyping was performed by indirect immunofluorescence and flow cytometry, using a panel of monoclonal antibodies (16). Each line was found to display the features of the leukemia cells of the respective patient. Concordance between RNA expression ratio (see below) and cell surface expression of the gene was determined as follows. The RNA expression ratios for CD2, CD3, CD4, CD10, and CD19 were plotted, and in each case, inspection of the values showed clear demarcation into two groups, either <1 or low ratios (negative) and high ratios (positive). Protein expression was determined by immunofluorescence and scored as negative or positive as shown in Table 1. Concordance of results (positive or negative) for both tests was determined on 63 sets of paired data. The cytogenetic analysis was carried out according to standard protocols (24).

cDNA Microarrays and Image Analysis. Total RNA was prepared from cell lines harvested in the exponential growth phase. Fluorescently labeled cDNA was synthesized from 45 μg of RNA by oligo(dT)-primed polymerization in the presence of Cy5 dUTP, whereas control RNA consisting of an equal mixture derived from Raji and Jurkat cells was labeled with Cy3. The 13,826 human cDNAs included in the study were obtained under the Cooperative Research and Development Agreement with Research Genetics (Huntsville, AL). Gene names are according to build 138 of the Unigene human sequence collection (25). Hybridization and scanning of microarrays, as well as image analysis with DeArray, were performed as described previously (26, 27).

Statistical Analyses. The hierarchical clustering analysis was performed as described previously (28). For clustering, a set of genes was selected that was reliably measured (mean intensity in the reference channel >500 fluorescence units). This set was sorted according to the SD of the expression ratio. The 1,500 most variable genes were clustered (using average linkage and the Pearson correlation coefficient). To identify the genes differentially expressed between cell lines of T- *versus* B-lineage, we generated a weighted list of genes and tested it by the random permutation method as described previously (29). Briefly, to generate a weighted gene list, the genes with high-quality measurements were ranked according to the signal-to-noise statistic (1). Let $[m_t(g), s_t(g)]$ and $[m_b(g), s_b(g)]$ denote the means and SDs of the natural logarithm of the expression levels (fluorescence ratios) of the gene g for the T- and B-lineage samples, respectively. The weight for each gene is defined as $w(g,t/b) = |m_t(g) - m_b(g)|/[s_t(g) + s_b(g)]$. The sign of $m_t(g) - m_b(g)$ is positive if g is more highly expressed in the T-cell samples. A random permutation test was performed to determine whether a gene was significantly associated with distinguishing the two classes. We randomly permuted the class labels of the samples 10,000 times and for each gene calculated the probability α of obtaining a larger weight for a random permutation than for the separation of T- and B-lineage samples.

Table 2 Karyotypes of childhood ALL cell lines

Cell line	Karyotype	Genetic feature
T-ALL		
PER-117	53,add(X)(q24),Y,t(1;11;9)(p13;p11;p22),+3,+7,+8,add(8)(q24.3),−9,t(12;18)(q24,q11), add(14)(q32), +13,+15,+19,+mar1,+mar2	
PER-255	46,XY,t(7;10)(q32–q34;q24),t(9;12)(p22;p12–13)	*HOX11* expression
PER-487	46,XY	
PER-427	46,XX	
PER-550	47,XY,add(1)(p36),t(1;8)(q?25;q?24),del(4)(q12), add(4)(p14),add(4)(q21),add(7)(q32), add(12)(q24), del(14)(q22q32),add(20)(q13),+mar	*LM02* expression
PER-537	47,XY,+8	
pre-B ALL		
PER-145	45,XY,der(3)t(3;?)(q12;?),der(4)t(4;?)(p15.2;?),t(5;17)(q15;p13),der(7)t(7;8)(p13–14;q13–21), −8,?t(9;20;9)(p13;p12;q34), der(12)t(12;?)(p13;?)	*TEL-AML1* expression
PER-278	46,XY,der(9)t(1;9)(q23;p13),der(19)t(1;19)(q23;p13)	
PER-371	46,XY,der(16)t(1;16)(q2?1;p13), der(19)t(1;19)(q?13;p13)/46,X,−Y,+?der(1)t(Y;1)(q12; ?q21), add(11)(q21),der(19)t(1;19)	
B-ALL		
PER-377	46,XY,t(2;13)(p12;q34),del(7)(q11q21),?inv(14)(q11q24), der(17)t(8;17)(q11;p11)/47,XY,idem, t(1;20)(q32;q13),+19	*MLL-AF9* rearrangement
PER-495	46,XY,t(8;14)(q24;q32)	
Infant leukemia		
PER-485	47,XX,der(4)t(4;11)(q21;q23)add(4)(p16),+6,del(7)(p14), add(8)(q24.3),der(9)inv(9)(p11q12)del(9)(p24), der(11)t(4;11)(q21;q23)	
PER-490	46,XX,t(4;11)(q21;q23)/46,XX,t(4;11),dup(1)(q12q44)/46,XX,t(4;11),der(2)t(1;2)(q12;q37)	

Results

The panel comprised six T-ALL lines, and they represent various stages of differentiation, ranging from the very immature PER-117 line to several lines which express mature T-cell markers (Table 1). None of them expressed CD10; however, PER-427 cells were positive for MHC class II antigens. By Northern blot analysis, high *LMO2* expression was detected in cell line PER-550, and cell line PER-255 is known to express high levels of *HOX11* attributable to t(7;10)(q32-q34;q24) (Ref. 18; Table 2). Three lines were of pre-B phenotype (Tables 1 and 2). Two of them, PER-278 and PER-371, showed der(19)t(1;19), which is present in 20–25% of pediatric pre-B ALLs. Cell line PER-145 was derived from a specimen obtained in third relapse, and it displayed a very complex karyotype. The presence of the *TEL-AML1* fusion transcript in this line was verified by reverse transcription-PCR. On the basis of their expression of IgM on the cell surface, lines PER-377 and PER-495 were classified as mature B-cell ALL, and PER-495 cells exhibited a t(8;14)(q24;q32) (Tables 1 and 2). Two lines were established from infants, both containing chromosomal aberrations of the *MLL* locus at 11q23. Detailed analysis of the *MLL* gene in PER-377 cells revealed the presence of an *MLL-AF9* rearrangement (23).

Cy5-labeled cDNA from the cell lines was hybridized to cDNA microarrays containing 13,826 elements, and the signals were obtained relative to Cy3-labeled control cDNA. The microarray data will be available on the Internet.[3] The resulting expression profiles were first examined to determine whether they provided an accurate reflection of the corresponding protein levels, as assessed by immunophenotype analysis and summarized in Table 1. The extensive characterization of the cell lines by surface marker analysis allowed us to rigorously assess the quality of the recorded expression profiles, and the comparison of protein and RNA expression showed excellent agreement. Concordant results were obtained for 62 of 63 (98.4%) independent measurements on the expression of three T-lineage markers (CD2, CD3, and CD4) and two B-lineage markers (CD10 and CD19). The exception was the measurements for CD3 on one cell line, whereas the cell surface expression of all other markers on this line was in excellent agreement with RNA expression levels. We next examined the expression of genes expected to be up-regulated as a result of translocations (Table 2). In all cases, the genes were found to be highly expressed as determined by expression profile. Examples included the *HOX11* gene, which was discovered at the t(7;10)(q32-q34;q24) breakpoint in PER-255 cells (18). Similarly, high *LMO2* expression was recorded for PER-550 cells, both by microarray and Northern blot analysis. Cell lines PER-278 and PER-371 exhibit a t(1;19), and both showed high RNA levels of *PBX1* by profile. Cell line PER-495, which contains a t(8;14)(q24;q32), had an elevated level of *MYC*. Taken together, the correlation between expression determined by microarray analysis and independently assessed protein and RNA expression levels demonstrated the consistent quality of the expression profiles obtained.

To examine the patterns of expression observed across cell lines, we selected a subset of genes that was reliably measured (see "Materials and Methods"). This set of genes was subjected to hierarchical clustering using the average linkage method. The unsupervised clustering identified two major clusters with the T-cell leukemias in one and B-lineage leukemias in the other cluster (Fig. 1). The two most closely related cell lines were the pre-B lines PER-278 and PER-371, both known to contain t(1;19). Within the T-cell group, cell lines PER-537, PER-550, and PER-255 formed the tightest cluster.

[3] Internet address: http://research.nhgri.nih.gov/microarray/selected_publications.html.

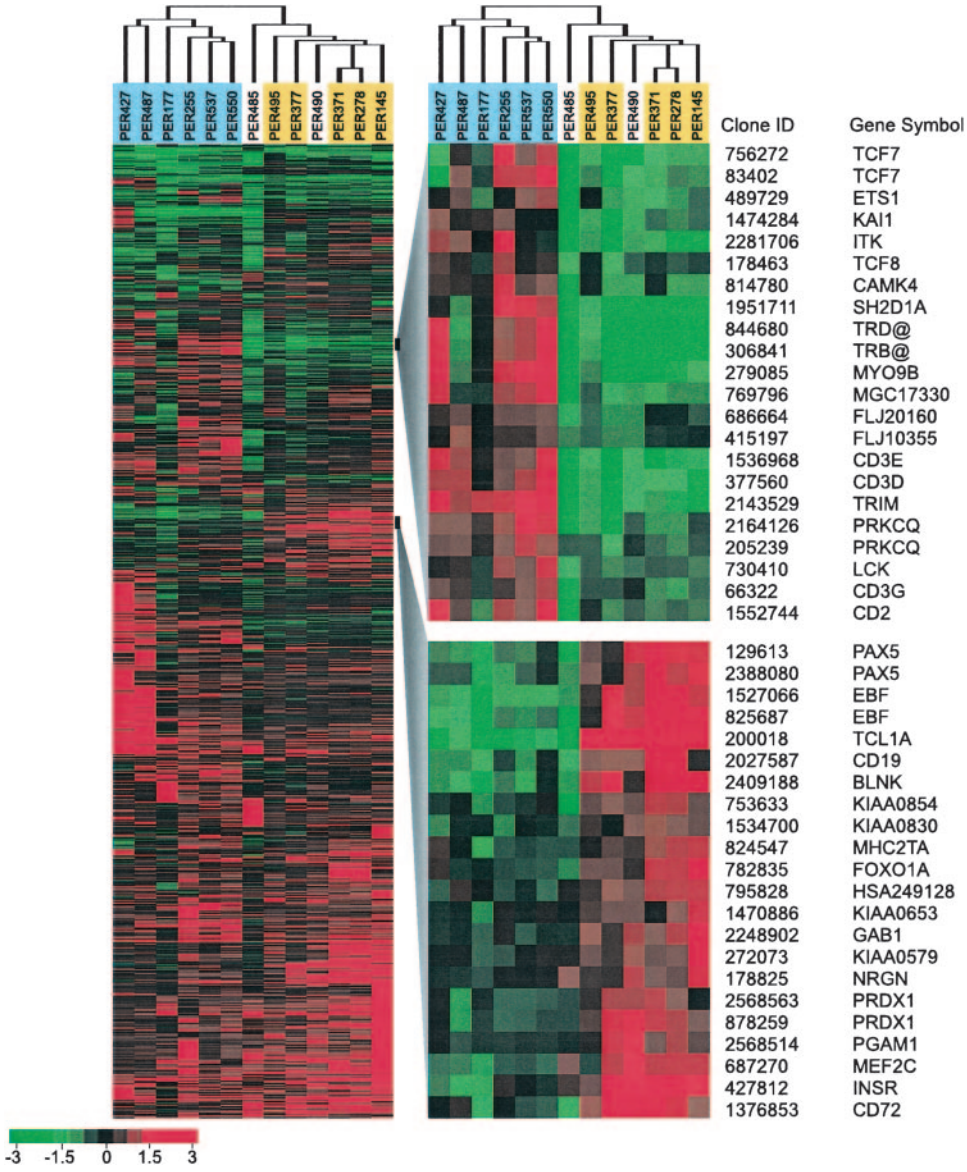

Fig. 1. Hierarchical clustering of expression data from pediatric leukemia cell lines representing 1500 array elements. *Right panels,* the T-ALL (*top*) and B-lineage ALL gene clusters (*bottom*). *Blue tag,* T-ALL; *yellow tag,* B-lineage ALL; *white tag,* infant leukemia. Log-transformed expression ratios are indicated according to the scale below.

The distinct clustering between cell lines of T- and B-lineage prompted us to identify the genes differentially expressed in the two clusters. A weighted list of genes which best discriminates between them was generated, and the top-scoring 50 genes are listed in Table 3. All of them showed a value of $\alpha < 0.003$, indicating the profound difference in gene expression between these two groups of leukemias, as already observed by hierarchical clustering (Fig. 1).

Discussion

The heterogeneity of pediatric ALL is well documented, yet the microarray technology has begun to provide detailed information on common features present in defined subsets of patients. The tight clustering of the two cell lines which display t(1;19) investigated here illustrates the fact that leukemias with the same translocations express similar sets of genes. Three recently published studies on primary patient specimens identified the molecular features defined by recurrent chromosomal translocations (13–15). Leukemia cells with translocations t(12;21)/*TEL-AML1* and t(1;19)/*E2A-PBX1* showed common GEPs, and patients with disease of theses subtypes are expected to have a good treatment outcome, particularly on current chemotherapy protocols. In contrast, patients with t(9;22)/*BCR-ABL* and t(4;11)/*MLL-AF4* are known to benefit from hematopoietic stem cell transplantation. Similarly, the known T-cell oncogenes *HOX11, TAL1,* and *LYL1* defined gene expression signatures among patients with T-ALL (12, 13), and differences in clinical outcome between these subgroups have been reported. The activation of key transcription factor genes or fusion genes was identified as the principal transforming event in the respective subgroups. In this study, established leukemia cell lines representing the distinct subtypes of ALL were investigated, because they are a great asset for future stud-

Table 3 Genes discriminating between ALL of T- and B-lineage

Weight	α	up(+)/down(−) in T *vs.* B	Clone ID	Gene
4.51595	0	−	2562279	Immunoglobulin heavy constant μ
3.72089	0.0024	+	247831	ESTs[a]
3.35546	0	−	246748	Bruton agammaglobulinemia tyrosine kinase
3.16278	0	−	241066	Homo sapiens cDNA: FLJ21028 fis, clone CAE07155
3.10326	0	+	2143529	T-cell receptor-interacting molecule
3.05368	0	−	845311	DKFZP566F084 protein
2.93712	0.0024	+	377560	CD3D antigen, δ polypeptide (TiT3 complex)
2.80172	0	−	2409188	B-cell linker protein
2.78049	0	−	2333826	MD-1, RP105-associated
2.66596	0	−	110281	Syntaxin 7
2.64287	0	−	1527066	Homo sapiens early B-cell transcription factor mRNA, partial cds
2.62345	0	−	2369226	Eukaryotic translation initiation factor 2, subunit 3 (γ, 52 kDa)
2.51444	0	−	214441	Immunoglobulin heavy constant μ
2.45823	0	+	2871658	T-cell activation, increased late expression
2.41246	0.0021	+	452023	Ubiquitin associated and SH3 domain containing, A
2.34441	0	−	366945	Spleen focus forming virus proviral integration oncogene spi1
2.34309	0	−	813390	ESTs, weakly similar to KIAA0766 [H. sapiens]
2.33976	0	+	462431	Ubiquitin-specific protease 20
2.32847	0	+	448068	Hypothetical protein FLJ10204
2.29914	0	−	825687	Early B-cell factor
2.28969	0	+	205239	Protein kinase C, θ
2.2831	0	+	2164126	Protein kinase C, θ
2.2639	0	+	1543346	Transketolase-like 1
2.20674	0	−	705274	Diacylglycerol kinase, δ (130 kDa)
2.19852	0	−	613251	DEAD-box protein abstrakt
2.10678	0.0024	+	2018154	Electron-transfer-flavoprotein, β polypeptide
2.07304	0.0023	+	840444	Homo sapiens cDNA: FLJ22439 fis, clone HRC09236
2.07092	0	−	878259	Peroxiredoxin 1
2.07001	0	−	505882	Hypothetical protein FLJ20550
2.06214	0	−	2507433	Suppressor of clear, *C. elegans*, homologue of
2.04142	0	+	208531	Hypothetical protein FLJ20551
2.03537	0.0024	−	472103	Suppressor of clear, *C. elegans*, homologue of
2.028	0	−	32229	Homo sapiens cDNA: FLJ23227 fis, clone CAE00645, highly similar to AF052138 Homo sapiens clone 23718 mRNA sequence
2.0209	0	−	129613	Paired box gene 5 (B-cell lineage-specific activator protein)
2.01773	0.0024	−	1561558	ESTs, highly similar to ITF2_HUMAN TRANSCRIPTION FACTOR 4 [H. sapiens]
1.98906	0.0023	+	1869186	CGI-39 protein; cell death-regulatory protein GRIM19
1.94572	0	+	725746	Signal transducer and activator of transcription 3 (acute-phase response factor)
1.93364	0	−	1635695	KIAA1080 protein; Golgi-associated, γ-adaptin ear containing, ARF-binding protein 2
1.91336	0	−	753633	KIAA0854 protein
1.9119	0.0023	+	462431	Ubiquitin-specific protease 20
1.91076	0	−	85624	Complement component 4 binding protein, α
1.90323	0.0021	−	810740	Immunoglobulin λ locus
1.89928	0	−	2548338	Diacylglycerol kinase, δ (130 kDa)
1.88614	0.0026	−	2215191	KIAA1032 protein
1.87044	0.0024	+	1636257	Dynactin 3 (p22)
1.86514	0.0019	+	810859	Natural killer cell transcript 4
1.84292	0.0024	+	357626	Fumarylacetoacetase
1.84084	0	+	2281706	IL-2-inducible T-cell kinase
1.83353	0	−	42214	Spleen tyrosine kinase
1.82543	0.0022	−	115281	CD79A antigen (immunoglobulin-associated α)

[a] EST, expressed sequence tag; ARF, ADP ribosylation factor; IL, interleukin.

ies to determine whether putative target genes can be exploited for therapeutic applications.

The suitability of cell lines for such studies critically depends on whether they display the identifying features for distinct disease subtypes. The large patient cohort study (14) singled out high expression of *PBX1* and *NID2* in leukemia cells with t(1;19), and these genes were highly expressed exclusively in the two cell lines which contain this translocation. Contrasting with all other cell lines, the *TEL-AML1*-expressing line PER-145 showed high expression of *PCLO* and *IDI1,* which is a prominent feature of leukemia cells with this translocation. The *HOX11*-positive cell line PER-255 revealed differential expression of *HOX11, CD1A, TYMS,* and *SCG2 versus* the other lines tested, confirming the data on patient specimens (13). *MEIS1* expression was reported to be exclusive to leukemia cells containing *MLL* rearrangements (14). Two infant leukemia cell lines analyzed here display t(4;11), and both expressed *MEIS1* at high levels. This feature was also present in cell line PER-377, which does not have a cytogenetically detectable *MLL* aberration,

yet is known to contain a molecularly detectable *MLL-AF9* rearrangement (23). Hence, all three lines known to contain *MLL* rearrangements displayed the distinguishing feature of high *MEIS1* expression. Taken together, the data on the panel of lines investigated here clearly demonstrated the presence of identifying features of ALL subtypes.

The search for genes that discriminate between ALL of T- and of B-lineage yielded two top-scoring genes for T-ALL, *CD3D* and T-cell receptor-interacting molecule *TRIM,* confirming the report by Yeoh *et al.* (14), who identified *CD3D* as the single gene required for class assignment to T-ALL. Similarly, among the discriminating genes highly expressed in B-lineage ALL, several genes coding for B-cell receptor signal-transducing elements were identified, including *IGM, SLP65,* and *CD79A,* confirming the findings by Yeoh *et al.* (14). Furthermore, the *PBX1* gene was the other gene reported by Yeoh *et al.* as sufficient for accurate assignment to the subclass of *E2A-PBX1* ALL, and this study confirmed very high expression of *PBX1* exclusively in the two cell lines that contain this translocation. Interestingly, in addition to the known recurrent rearrangement der(19)t(1;19)(q23;p13), one of the cell lines, PER-278, showed a der(9)t(1;9)(q23;p13), which results in monosomy 9p and trisomy 1q. This combination of abnormalities has been reported in four cases of ALL in the literature, although in several cases, the t(1;9) breakpoints are slightly different from those in our cell line (30–33). There are also two reports of a similar der(9)t(1;9) rearrangement not associated with der(19)t(1;19)(q23;p13) in ALL (34, 35).

The identification of specific, frequently occurring molecular alterations in cancer cells is expected to fundamentally change the diagnosis and treatment of all cancers. Drugs like Herceptin for the treatment of metastatic breast cancer and Gleevec for the therapy of patients with t(9;22) chronic myelogenous leukemia are the first examples of gene-based cancer drugs. The large-scale studies on pediatric ALL patients have identified genes which may prove to be useful targets against which novel therapeutic agents could be developed (13–15). Genes particularly suited for drug discovery purposes are those expressed at elevated levels in cancer cells. The relationship between GEPs and sensitivity to chemotherapeutic agents in 60 National Cancer Institute tumor cell lines exemplified how variations in the transcript levels of particular genes relate to mechanisms of drug sensitivity and resistance (3, 4). Genes of interest identified in GEPs would need to be subjected to additional experiments to determine the biochemical mechanisms through which such genes influence malignant growth and establish their causative role in the process. Studies of this kind also depend greatly on the availability of well-characterized cell lines. The data presented here on a panel of pediatric leukemia cell lines demonstrate excellent concordance between RNA data and expression of proteins, a feature highly relevant for the design of novel drugs. As evidenced by the successful development of target-directed therapies, the efficiency of new agents needs to be assessed in model systems, initially in cell lines, followed by studies in animals. Hence, suitable cell lines are indispensable for the development of novel gene-based drugs for future cancer therapy.

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