Identification of genes associated with chemotherapy crossresistance and treatment response in childhood acute lymphoblastic leukemia

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Summary

Acute lymphoblastic leukemia (ALL) can be cured with combination chemotherapy in over 75% of children, but the cause of treatment failure in the remaining patients is unknown. We determined the sensitivity of ALL cells to individual antileukemic agents in 441 patients and used a genome-wide approach to identify 45 genes differentially expressed in ALL exhibiting crossresistance to prednisolone, vincristine, asparaginase, and daunorubicin. We also identified a distinct phenotype of discordant resistance to asparaginase and vincristine and 139 genes whose expression was associated with this novel phenotype. The expression of these genes discriminated treatment outcome in two independent patient populations, identifying a subset of patients with a markedly inferior outcome (37% ± 13% 5 year DFS).

Introduction

Pediatric acute lymphoblastic leukemia (ALL) has long served as a paradigm for development of curative chemotherapy of disseminated cancer, with contemporary treatment protocols achieving long-term disease-free survival (DFS) of 80% (Pui and Evans, 1998; Pui et al., 2002). This success is, in part, related to modulating treatment intensity to each patient's risk of relapse. Although clinical features (e.g., age, leukocyte count at diagnosis) and biological characteristics of leukemia cells (e.g., chromosome number or translocations) are useful prognostic variables to guide treatment intensity, many patients with good features are not cured and vice versa. Intrinsic (de novo) and acquired resistance to multiple antineoplastic agents represent major obstacles for successful treatment of the remaining 20% of patients who are not cured with current therapy (Pui and Evans, 1998; Sonneveld, 2000).

Cellular drug resistance measured in vitro as lethal concentration (LC_{50}) to prednisolone (PRD), vincristine (VCR), and asparaginase (ASP) in primary leukemic cells isolated from patients at diagnosis is significantly associated with DFS in children with ALL (den Boer et al., 2003; Hongo et al., 1997; Kaspers et al., 1997; Pieters et al., 1991). The existence of crossresistance patterns between structurally distinct antileukemic agents (Kaspers et al., 1998) suggests that common pathways may contribute to cellular drug resistance in pediat-

SIGNIFICANCE

Many children with "good risk" molecular subtypes of acute lymphoblastic leukemia (ALL) are not cured with current treatment, for reasons that remain largely unknown. To elucidate genomic determinants of treatment outcome, we used a genome-wide approach to identify genes and gene expression patterns associated with de novo multiple drug resistance of leukemia cells. This revealed 45 genes associated with crossresistance to four mechanistically distinct antileukemic agents and 139 genes significantly related to a novel phenotype of discordant resistance to vincristine and asparaginase. The expression pattern of these genes was also significantly related to treatment outcome. These findings provide insights to the biological basis of de novo multiple drug resistance and illuminate potential targets for overcoming this cause of treatment failure in childhood ALL.

ric ALL (Pieters and den Boer, 2003). However, there is a paucity of data on genomic determinants of de novo crossresistance to ALL chemotherapy.

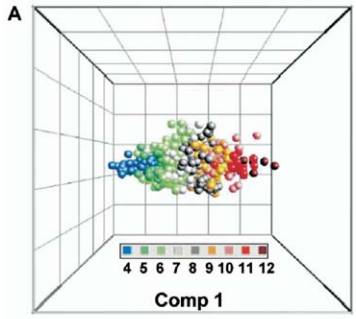
Acquired multiple drug resistance is a cancer phenotype frequently associated with increased drug efflux mediated by transmembrane transporters (e.g., *ABCB1*, *ABCB4*, *ABCC1*, *ABCG2*, and *MVP*). This acquired phenotype can be induced in vitro by prolonged exposure to low drug concentrations but is not a common mechanism of de novo drug resistance in childhood ALL (den Boer et al., 1998; Pieters and den Boer, 2003; van den Heuvel-Eibrink et al., 2000). Also, drugs that are not transported by these efflux pumps, such as PRD, dexamethasone, and ASP, are essential components of ALL treatment protocols. Therefore, other mechanisms of multiple drug resistance must be operative when ALL cells exhibit de novo crossresistance to these agents.

Gene expression profiles were shown to discriminate drug sensitivity to 232 compounds in the NCI panel of 60 human cancer cell lines (Staunton et al., 2001) and to nine drugs in 85 human tumor xenografts in mice (Zembutsu et al., 2002). More recently, the expression of 172 human gene probe sets was shown to discriminate primary ALL cells that are sensitive or resistant to one of four antileukemic agents (Holleman et al., 2004). Little is known, however, about genes that confer de novo crossresistance of primary ALL cells to multiple anticancer agents, a phenotype likely associated with a very poor prognosis. The current study was therefore undertaken to determine whether genes could be identified that are differentially expressed in primary B lineage ALL cells that exhibited de novo crossresistance to two or more widely used antileukemic agents (i.e., PRD, VCR, ASP, and daunorubicin [DNR]). The prognostic importance of genes associated with crossresistance was also assessed by determination of their relation to treatment outcome in two independent patient populations.

Results

Definition of the multiple drug resistance phenotype

De novo sensitivity of leukemia cells to four widely used antileukemic agents was significantly related among 441 children with diagnosed B lineage ALL (Figure S1 in the Supplemental Data available with this article online). Principal component analysis (PCA) was used to reduce the dimensionality of multivariate data (four LC_{50} values for 441 patients) by transforming the original variables into uncorrelated ones (i.e., the principal components) that account for decreasing proportions of variance (Figure 1). Most of the variation was preserved by the first component (48%), indicating common variation among all four drugs, with the four coefficients being similar in weight and direction. The second component explained 24% of the variation in LC_{50} values, affecting VCR and ASP by the same weight but in opposite directions, revealing discordant sensitivity between these two drugs (Figure 1). Using the weight of the first and second component, respectively, a crossresistance score (CR score) and a vincristine-asparaginase score (VCR-ASP score) was computed for each patient. The Spearman's rank correlations between the CR score and the LC_{50} values for each drug were all over 0.5 with $p < 0.0001$ (except for VCR [rho = 0.23, p = 0.01]). The top and bottom quartiles of CR scores for the 441 patients were used to define patients with cross-sensitive and crossresistant ALL. Likewise, the top and bottom quartiles of the VCR-ASP score defined two cohorts of patients exhibiting discordant resistance to VCR and ASP (i.e., ALL cells that were VCR sensitive plus ASP resistant, or those VCR resistant plus ASP sensitive, respectively). For the 129 patients whose ALL cells were analyzed for gene expression,



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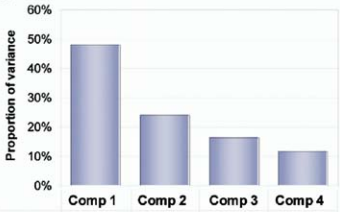

	Comp 1	Comp 2	Comp 3	Comp 4
PRD	0.544	-0.121	0.714	0.425
VCR	0.428	0.683	-0.444	0.392
ASP	0.394	-0.715	-0.541	0.202
DNR	0.604	0	0	-0.791

Figure 1. Principal component analysis and LC_{50} value coefficients

Principal component analysis (PCA) based on the LC_{50} value coefficients for four antileukemic agents in primary ALL cells from 441 patients with B lineage ALL. Principal component plot, where each sphere represents a different ALL sample, and the location is based on the first three principal components using the patients' LC_{50} values for the four antileukemic agents. In **A**, ALL samples are labeled (see color key) according to the sum of the LC_{50} drug resistance score as previously described (Pieters et al., 1991) (i.e., 1 for sensitive, 2 for intermediate, 3 for resistance per agent; the sum results in scores of 4 [most cross-sensitive] to 12 [most crossresistant]). In **B**, ALL samples are labeled (see color key) according to the asparaginase (ASP) and vincristine (VCR) discordant sensitivity phenotype. **C** illustrates percent variance in LC_{50} values explained by each component of the PCA. Each component was computed as a linear combination of the LC_{50} values for the four drugs, with the coefficients listed in the bottom panel. For each component (each column), a positive coefficient indicates a positive correlation between the LC_{50} values of the drug and the computed component scores. A drug with higher absolute coefficient value contributes more to the component and is more correlated with the component scores.

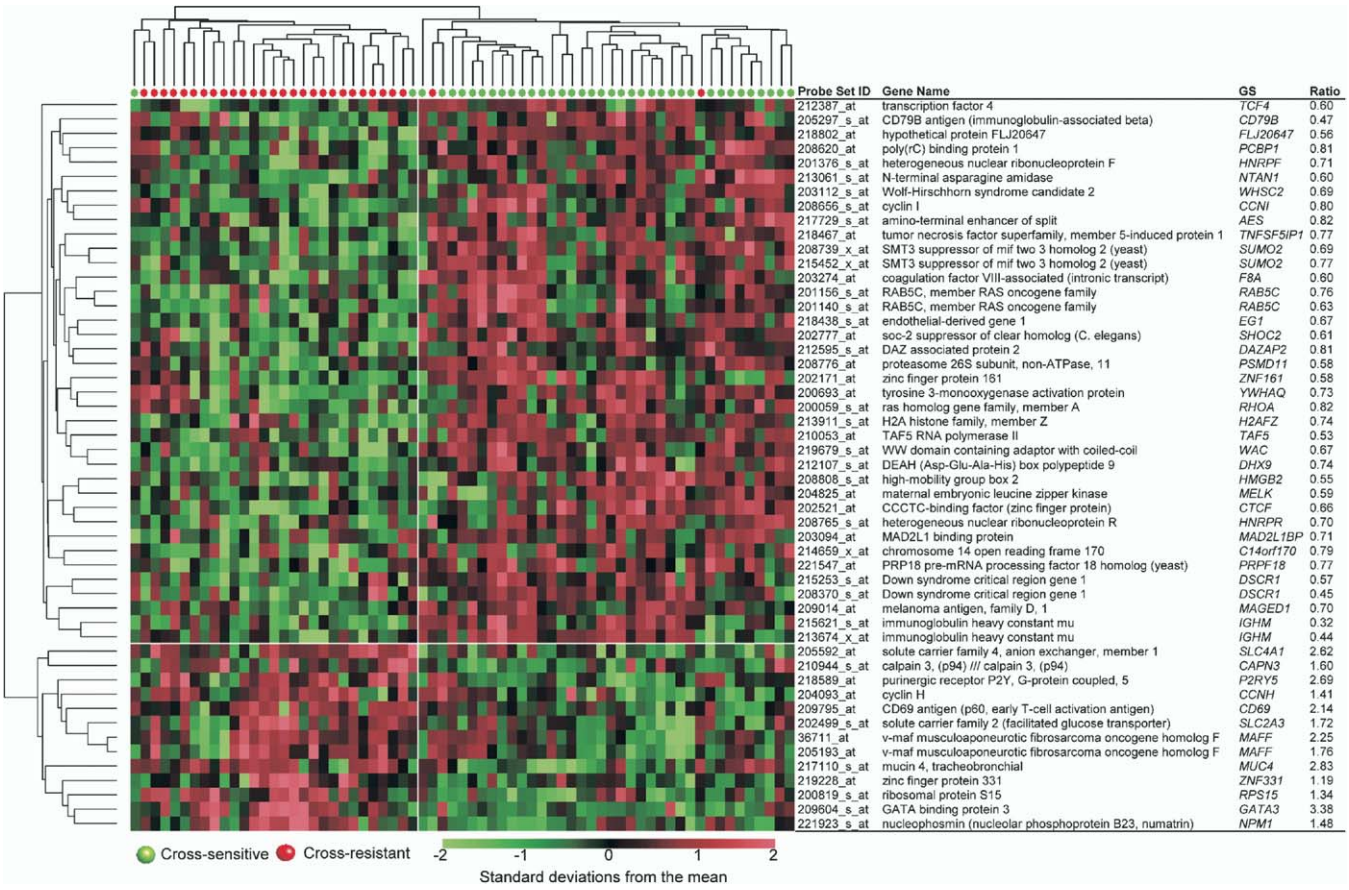

Figure 2. Hierarchical clustering using genes that discriminate crossresistant and cross-sensitive B lineage ALL patients

Hierarchical clustering using 51 significant probe sets (45 genes and one cDNA). Each column represents an ALL patient labeled across the top with green circles for cross-sensitive patients (n = 38) and with red circles for crossresistant patients (n = 29). Each row represents a probe set, with the probe set ID, gene name, gene symbol (GS), and ratio of expression listed for each probe set. The ratio of expression is the median expression level in crossresistant patients divided by the median level in cross-sensitive patients (ratio > 1 indicates overexpression in crossresistant patients, whereas a ratio <1 indicates underexpression in crossresistant patients). The "heat map" indicates high (red) or low (green) level of expression relative to the scale shown.

crossresistance and VCR-ASP discordant resistance phenotypes (CR scores, VCR-ASP scores) were assigned based on the values used to define these phenotypes in the entire population of 441 patients. Figure S2 shows that the distribution of CR scores and VCR-ASP scores in the subset of patients for whom gene expression was performed (n = 129) did not differ from the entire patient population (n = 441). Figure S3 shows the distribution of LC_{50} values for each drug for patients classified according to crossresistance (CR score) or discordant resistance to VCR and ASP (VCR-ASP score).

Crossresistance was significantly related to patient age at diagnosis, with older patients being more likely to exhibit a crossresistant phenotype (Table S1; p = 0.002, R = 0.27, Pearson correlation). The VCR-ASP discordant resistance was not related to patient age (p = 0.12, R = 0.14, Pearson correlation), although there was a tendency for older children to be VCR sensitive plus ASP resistant. No statistically significant difference in sex or proportion of patients with white blood cell counts at diagnosis lower than 50/nl (WBC < 50/nl) was detected between crossresistant and cross-sensitive ALL or between the two VCR-ASP discordant resistance groups.

Genes differentially expressed in crossresistant ALL

We identified 51 gene probe sets (representing 45 different known genes and one cDNA clone) that were differentially expressed in crossresistant and cross-sensitive ALL based on correlation analysis with the CR score (p < 0.0001; false discovery rate [FDR] = 2.8%; Table S2). The 10-fold crossvalidation using Spearman's rank correlation with the top 51 probe sets revealed significant concordance with the observed CR gene expression score (rho = 0.52; p < 0.0001; Table S3). Hierarchical clustering using the selected probes sets correctly assigned 63 of 67 crossresistant and cross-sensitive cases (Figure 2). Similarly, when 51 gene probe sets were used, the PCA correctly clustered the great majority of patients into either the crossresistant cluster or the cross-sensitive cluster (Figure S4A). The biological functions of 32 of the 51 genes discriminating crossresistance are annotated in the Gene Ontology (GO) database (Figure S5), with a total of 55 annotations (some genes are assigned to multiple functions). Genes involved in nucleic acid metabolism, including transcription (most genes), RNA and DNA processing, and DNA repair, were significantly overrepresented in the group of genes discriminating crossresistance, accounting for 26% of the crossresistance genes (14

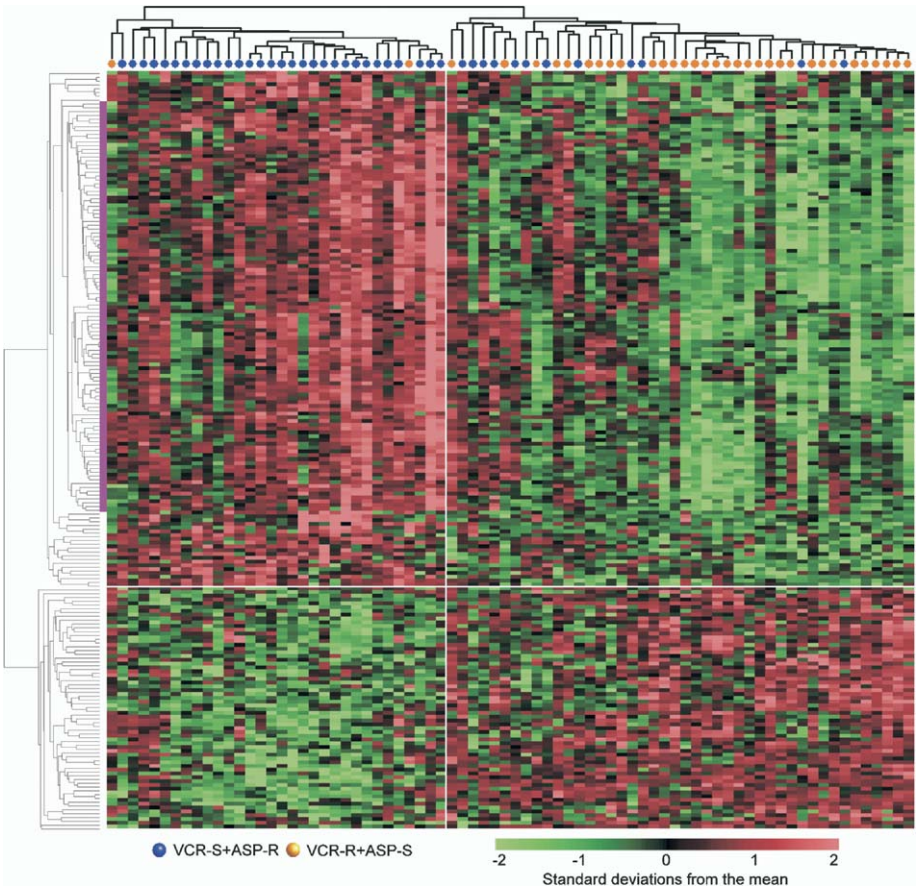

Figure 3. Hierarchical clustering of genes discriminating discordant resistance to VCR and ASP

Hierarchical clustering using 200 probe sets (139 genes, 13 cDNAs). Each column represents an ALL sample labeled across the top with blue circles for VCR-sensitive plus ASP-resistant ALL (VCR-S+ASP-R; n = 42) and with orange circles for VCR-resistant plus ASP-sensitive ALL (VCR-R+ASP-S; n = 34). Each row represents a probe set, and the probe set ID, gene name, gene symbol (GS), and ratio of expression are provided in Table S5. The cluster of genes highlighted in magenta contains genes predominantly involved in protein biosynthesis (97 out of 108). The "heat map" indicates high (red) or low (green) level of expression according to the scale shown.

out of 55) compared to 14% of genes on the entire array (p = 0.013).

Genes discriminating discordant resistance to VCR and ASP

Based on correlation analysis with the second component of the PCA, we identified 200 probe sets, representing 139 different genes and 13 cDNAs, that discriminated patients whose ALL cells were VCR sensitive plus ASP resistant (n = 42 patients) versus VCR resistant plus ASP sensitive (n = 34 patients; $p < 0.00006$; FDR = 6.3%). The genes discriminating VCR-ASP discordant resistance, their Affymetrix ID, gene symbol, and expression ratio are provided in Table S5. Hierarchical clustering (Figure 3) correctly grouped 62 of the 76 patients whose VCR-ASP scores fell into the top or bottom quartiles. PCA depicts the clustering of these patients based on the 200 probe sets that were associated with discordant resistance to VCR and ASP (Figure S4B).

Genes involved in protein biosynthesis (90 of 200 probe sets) were overrepresented among genes discriminating discordant resistance to VCR and ASP. These genes included ribosomal proteins (77 of 200 probe sets) and translation elongation factor-related genes (15 of 200 probe sets). These genes were tightly clustered in one branch of the hierarchical clustering and were expressed at a higher level in VCR sensitive plus ASP resistant ALL compared to VCR resistant plus ASP sensitive ALL. The majority of other functional categories (e.g., transport, cell communication, response to endogenous stimulus) were underrepresented among the discriminating genes, compared to the entire genome (Figure S5).

Because protein synthesis can be altered by various mechanisms, we compared expression levels of *RAS-MAPK* pathway genes, *p53*, *RB*, n-*myc*, c-*myc*, *cyclin D*, *PTEN*, *AKT*, and *mTOR/FRAP*, none of which were expressed at a significantly different level in ALL cells that were VCR sensitive plus ASP resistant versus VCR resistant plus ASP sensitive.

To address the question of general coregulation of protein synthesis genes, we identified all probe sets on the U133A microarray that are annotated in the GO database as involved in the biological process of "protein synthesis" (i.e., 331 probe sets; 200 unique genes). Of these, only 31% (104 probe sets representing 59 genes) were related to VCR-ASP discordant resistance by cluster analysis. Of these, 58% (34 of 59 genes) or only 17% of protein synthesis genes were significant in the VCR-ASP discordant resistance analysis.

Because ribosomal proteins are expressed at higher levels in VCR-sensitive and ASP-resistant ALL, we have computed the 95% confidence intervals (CI) for the ratios of the VCR-sensitive plus ASP-resistant versus VCR-resistant plus ASP-sensitive ALL cells for each of the ribosomal protein genes (77 gene probe sets) that differed significantly ($p = 0.00119$ to <0.0001, Student's t test) between these two VCR-ASP discordant resistant phenotypes (42 VCR-S + ASP-R versus 34 VCR-R + ASP-S). The 95% CI for the observed ratio for each

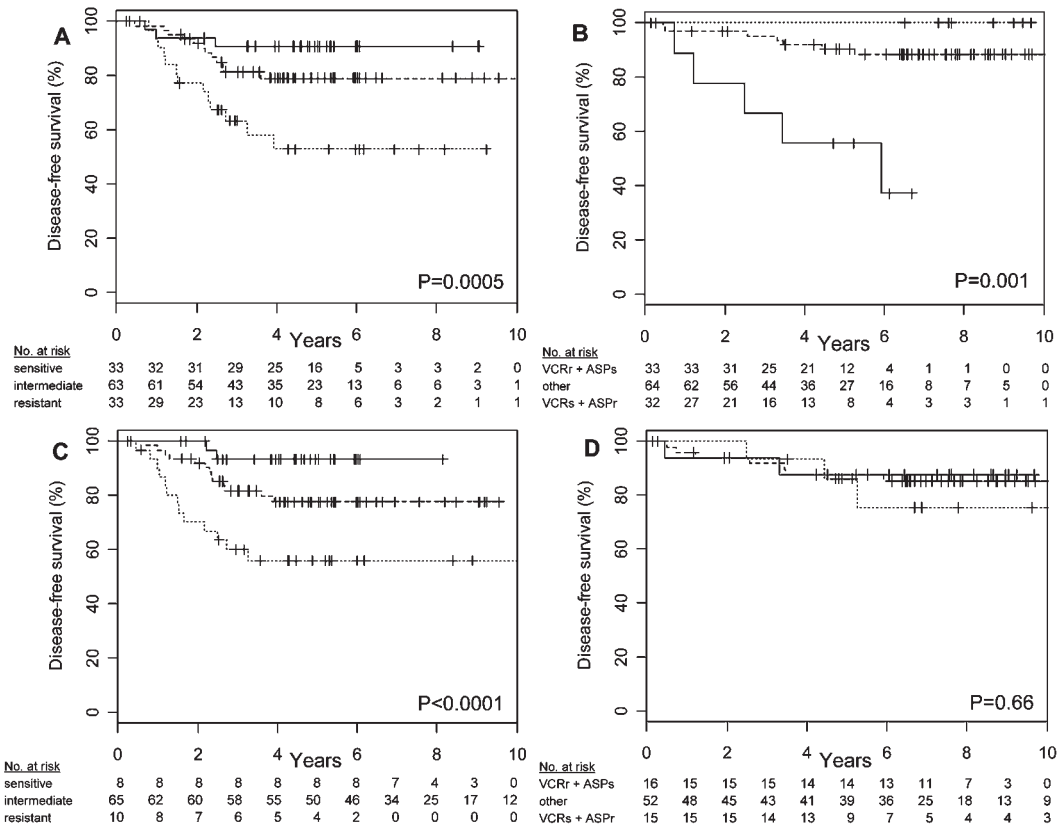

Figure 4. Disease-free survival among patients with crossresistant versus cross-sensitive B lineage ALL and patients with discordant VCR-ASP sensitivity

A and **C** depict disease-free survival (DFS) among patients with B lineage ALL based on different gene expression cross-sensitivity scores (CR score). **A** represents the 129 COALL/DCOG patients (crossresistant [n = 33], intermediate [n = 63], and cross-sensitive [n = 33]), and **C** represents the 83 St. Jude patients (n = 8, n = 65, and n = 10, respectively). **B** and **D** depict DFS among patients with B lineage ALL based on different gene expression scores for discordant sensitivity to vincristine and asparaginase (VCR-ASP score). **B** represents the 129 COALL/DCOG patients (VCR sensitive plus ASP resistant [VCR-S+ASP-R], n = 32; intermediate, n = 64; VCR resistant plus ASP sensitive [VCR-R+ASP-S], n = 33), and **D** represents the 83 St. Jude patients (n = 15, n = 52, n = 18, respectively).

significant ribosomal protein gene always exceeded 1.0 (i.e., lowest 95% CI 1.13 to 1.55, variance for the ratio <3%).

Multiple drug resistance in specific subtypes of B lineage ALL

ALL with the *TEL-AML1* or *E2A-PBX1* translocation or hyperdiploid ALL was more likely to be cross-sensitive (Table S1). ALL with *TEL-AML1* or hyperdiploidy were also more likely to have a favorable VCR-ASP discordant resistance phenotype (Table S1).

Altogether, 31 of 34 (91%) ASP-sensitive plus VCR-resistant ALLs were either *TEL-AML1* or hyperdiploid ALL ($p < 0.0001$, Fisher's exact test). Notably, however, only 13 (11 genes) of the 200 probe sets that discriminate discordant resistance to VCR and ASP also discriminate *TEL-AML1* or hyperdiploidy from other ALL subtypes (with top 100 probe sets for each) using genes that we have previously reported as discriminating these genetic subtypes (Table S7) (Ross et al., 2003; Yeoh et al., 2002).

Hierarchical clustering using genes that discriminated the crossresistance phenotype did not group ALL cases by molecular or lineage subtypes (Figure S6A). However, hierarchical clustering using the 200 probe sets that discriminated VCR-ASP discordant resistance grouped patients based on ALL subtype, in particular hyperdiploid and *TEL-AML1*, were grouped as ASP sensitive and VCR resistant, whereas *E2A-PBX1*, *MLL-AF4*, and *BCR-ABL* were primarily grouped as VCR sensitive and ASP resistant (data not shown). Therefore, to assess potential subtype bias in genes discriminating VCR-ASP discordant sensitivity, we adjusted the second component (VCR-ASP score) for the genetic subtypes by linear model and by analyzing the residuals identified 40 probe sets, the expression of which were significantly related to the adjusted VCR-ASP scores (33 different genes, 3 cDNAs; $p < 0.0008$; FDR = 29.5%; Figures S6B and S6C). Twenty-four (60%) of these probe sets were among the 200 probe sets discriminating VCR-ASP discordant resistance. Similar to the analysis without adjusting for ALL genetic subtype, 53% (21 of 40) of these genes encode ribosomal proteins (Figure S7).

Comparison with single drug resistance gene expression patterns

We previously identified gene expression patterns that discriminate ALL cells exhibiting resistance to *individual* antileukemic agents: PRD (33 genes, 3 cDNAs), VCR (40 genes, 14 cDNAs), ASP (35 genes, 10 cDNAs), and DNR (20 genes, 2 cDNAs) (Hol-

Table 1. Multivariable Cox proportional hazards regression analysis of the risk of relapse in relation to known prognostic factors, crossresistance, and VCR-ASP discordant resistance gene expression scores

	COALL/DCOG Patients				St. Jude Patients			
Variable	n	HR	95% CI	p value	n	HR	95% CI	p value
Age								
<10 years	96	1.0[a]			57	1.0[a]		
>10 years	33	1.57	0.63–3.9	0.34	26	1.3	0.29–5.9	0.73
WBC								
<49/nl	91	1.0[a]			50	1.0[a]		
50–100/nl	20	0.75	0.2–2.75	0.66	16	1.84	0.27–12.18	0.53
>100/nl	18	2.71	1.07–6.83	0.035	17	1.68	0.27–10.38	0.57
ALL subtype								
B-other	42	1.0[a]			27	1.0[a]		
BCR-ABL	5	2.9	0.74–11.4	0.13	8	35.0	4.55–270	0.0006
E2A-PBX1	6	1.19	0.24–5.8	0.83	12	1.73	0.14–21.1	0.67
Hyperdiploid	33	0.48	0.11–2.13	0.33	15	2.68	0.21–34.4	0.45
MLL-AF4	3	21.9	3.85–124.2	0.0005	5	6.77	0.74–61.6	0.09
TEL-AML1	40	0.19	0.04–0.99	0.049	16	0.92	0.05–15.5	0.95
GE score CR[b]	129	1.03	0.99–1.08	0.16	83	1.28	1.07–1.52	0.0075
GE score VA[b]	129	1.00	0.99–1.01	0.65	83	0.99	0.97–1.01	0.35

Multivariable Cox regression analysis computed with known prognostic factors (i.e., white blood cell count [WBC]), age at diagnosis, ALL subtype, and both gene expression scores as a continuous variable for COALL/DCOG and St. Jude patients. Number of patients (n), hazard ratios (HR), p values, and 95% confidence intervals (95% CI) are shown.

[a] Reference group.

[b] Using the gene expression score as a continuous variable. CR, cross-resistance; VA, VCR-ASP.

leman et al., 2004). Of the 108 unique known genes and 28 cDNA clones (146 probe sets) discriminating single drug resistance, only 15% of genes (16 genes, 18 probe sets) were also among genes discriminating crossresistance; the overlap of multiple drug crossresistance and single drug resistance was 12 genes (13 probe sets) for PRD, five genes for DNR, three genes (four probe sets) for ASP, and no genes for VCR (four of these 22 probe sets were duplicates; Table S8). The overlap of genes discriminating VCR-ASP discordant resistance and single drug resistance to VCR was 11 genes and three cDNA clones (15 probe sets), and that to ASP was 28 genes and two cDNA clones (39 probe sets). There was no overlap in genes discriminating VCR-ASP discordant resistance and genes discriminating PRD or DNR single drug resistance (Table S9). For all overlapping genes, the level of expression (over- or underexpressed) associated with resistance was concordant between the single and multiple drug resistance analyses.

Gene expression and treatment outcome

The median follow-up for the 129 patients included in the gene expression analysis was 4.3 years from diagnosis. Crossresistant patients (i.e., bottom quartile of the gene expression CR scores) had significantly worse 5 year DFS compared to patients classified as cross-sensitive (53% ± 10% versus 91% ± 5%; hazard ratio [HR] = 5.99; p = 0.005; Figure 4A and Table S10A), with the remaining patients having an intermediate outcome (80% ± 5%). Among patients whose ALL cells were ASP sensitive plus VCR resistant, 5 year DFS was 93% ± 5%, whereas 5 year DFS was only 56% ± 9% (HR = 9.04; p = 0.0038; Figure 4B and Table S10A) among patients whose ALL was VCR sensitive plus ASP resistant. The remaining group had an intermediate 5 year DFS of 78% ± 6%. The 5 year DFS of 17 patients with the most unfavorable quartile for both CR and VCR-ASP gene expression scores was 37% ± 13%, compared to 85% ± 10% in 14 patients in the best quartile for both gene expression scores (Figure S9; HR = 6.05; p = 0.0008).

The predictive value of these gene expression scores (CR and VCR-ASP) was tested in an independent cohort of 83 B lineage ALL patients who had been treated with these medications, but according to a different protocol (Total Therapy 13) at St. Jude Children's Research Hospital. The patients in this population defined as cross-sensitive based on their CR gene expression score had a significantly better outcome (5 year DFS = 100% ± 0%) than patients with the crossresistant gene expression score (5 year DFS = 56% ± 17%; HR = 7.76; p = 0.0005; Figure 4C and Table S10B), similar to that observed in the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL)/Dutch Childhood Oncology Group (DCOG) patient population (91% versus 53%). In contrast, DFS was not significantly different for VCR-resistant plus ASP-sensitive ALL compared to VCR-sensitive plus ASP-resistant ALL, for patients treated on the St. Jude protocol (HR = 1.67; p = 0.57; Figure 4D and Table S10B).

Single factor Cox regression analysis indicated that gene expression scores for crossresistance (high scores indicate crossresistance; low scores indicate cross-sensitivity) and VCR-ASP discordant resistance (high scores indicate VCR sensitive + ASP resistant; low scores indicate VCR resistant + ASP sensitive) were each significantly related to DFS in the study cohort. In the univariate analyses, the crossresistance gene expression score was the only variable significantly related to treatment outcome in both patient cohorts (HR = 5.99, p = 0.0053 [COALL/DCOG]; HR = 7.76, p = 0.0005 [St. Jude]; Table S10A). Additional variables that were significant in one cohort, but not the other, included WBC, *MLL-AF4* fusion, *TEL-AML1* fusion, and VCR-ASP score in the COALL/DCOG population, and *BCR-ABL* fusion in the St. Jude cohort.

The cohort of St. Jude patients showed a similar trend for the CR gene expression score (HR = 7.76; p = 0.0005; Table

S10B), whereas the VCR-ASP gene expression score was not significantly related to treatment outcome in the St. Jude cohort ($p > 0.5$). Of note, in the St. Jude cohort ($n = 83$) there were no relapses in children with a low CR score; thus, no HR could be determined for the low CR-group. Nevertheless, the high CR gene expression score was significantly related to an increased risk of relapse when compared to the intermediate CR score.

The favorable VCR-ASP scores were predominantly in ALL cells with either the *TEL-AML1* fusion or hyperdiploidy, and the unfavorable scores were predominantly in cells with the *BCR-ABL* or *MLL-AF4* fusion. The strongest contributing predictor in the COALL/DCOG cohort in a multivariable Cox regression analysis that included all known prognostic factors and both gene expression scores as continuous variables was presence of the *MLL-AF4* fusion, WBC > 100/nl, and presence of the *TEL-AML1* fusion ($p = 0.0005$, $p = 0.035$, $p = 0.049$, respectively; Table 1), followed by the CR score ($p = 0.16$). The CR score was the strongest contributing predictor, other than presence of the *BCR-ABL* gene fusion in a multivariable Cox regression analysis in the St. Jude cohort ($p = 0.0075$; Table 1). The low VCR-ASP gene expression score was not significantly related to outcome when compared to the high VCR-ASP score ($p = 0.35$; Table 1).

The worst group (bottom 33%) defined by PRD, VCR, ASP, DNR (PVAD) combined single drug resistance score (5 year DFS: 60% ± 9% [COALL/DCOG; $n = 127$]; 68% ± 11% [St. Jude; $n = 92$]) was used to test whether the crossresistance gene expression pattern (score) can further significantly discriminate patients at higher and lower risk for relapse (Figure 5). This identified a subgroup that has a significantly worse outcome, beyond that predicted by the worst single drug resistance gene expression pattern in two independent cohorts of patients. Patients with high CR scores had a significantly worse 5 year DFS (40% ± 14% [COALL/DCOG; $n = 41$; $p = 0.044$]; 50% ± 16% [St. Jude; $n = 19$; $p = 0.048$]) compared to patients with low scores (cross-sensitive, 76% ± 11% [COALL/DCOG], 89% ± 11% [St. Jude]). Consistent with findings in the entire cohort of St. Jude patients, the VCR-ASP discordant resistance score further discriminated patients at higher risk and lower risk in the COALL/DCOG cohort (87% ± 9% versus 27% ± 14%; $p = 0.001$), but not in the St. Jude cohort (Figure S9).

Crossresistance genes also discriminate mercaptopurine resistance

To assess whether the identified crossresistance gene expression profile is related to resistance to a broader spectrum of antileukemic agents (thus a more general multiple drug resistance phenotype), we used in vitro sensitivity for mercaptopurine. The crossresistance gene expression score was significantly predictive of in vitro resistance of mercaptopurine ($n = 51$; $p = 0.007$, Spearman's rank correlation), tested at St. Jude in ALL cells from patients enrolled on the Total Therapy 15 protocol, in addition to mercaptopurine sensitivity tested in children of the COALL/DCOG protocols ($n = 29$; $p = 0.0002$; Table S11). Notably, mercaptopurine resistance was not related to the VCR-ASP discordant resistance gene score ($p = 0.54$; $p = 0.13$), and more importantly, no significant association was found for mercaptopurine resistance with the combined single drug resistance gene expression scores (PVAD; $p = 0.99$; $p = 0.06$) (Holleman et al., 2004) These data indicate that the crossresistance gene expression score (CR) is significantly predictive of in vitro resistance of other mechanistically distinct antileukemic agents beyond the four drugs investigated.

Discussion

Although the cure rate of children with ALL is approximately 80%, many patients are not cured with today's treatment (Chauncey, 2001; Pui et al., 2004a). Drug resistance is an important cause of treatment failure, but the responsible mechanisms are largely unknown (Pieters and den Boer, 2003). Several studies have shown the existence of crossresistance patterns among structurally related and structurally unrelated drugs in ALL (den Boer et al., 2003; Holleman et al., 2003; Kaspers et al., 1998; Holleman et al., 2004). The extensively studied multiple drug resistance mechanisms (e.g., *ABCC1* [*MRP1*], *MVP* [*LRP*], and *ABCB1* [*MDR1*]) are of limited importance in ALL, particularly as a mechanism for de novo drug resistance (den Boer et al., 1998; van den Heuvel-Eibrink et al., 2000). Thus, insights are needed to understand the genomic basis of de novo multiple drug resistance in ALL.

Genome-wide analysis of gene expression in human ALL cells represents a relatively unbiased approach to identify genomic determinants of drug response and drug resistance (Cheok et al., 2003; Holleman et al., 2004; Evans and Relling, 2004). In a previous study, we identified genes associated with resistance to *single* antileukemic agents, but not genes associated with multiple drug resistance (Holleman et al., 2004). Because crossresistance to multiple agents would likely forebode an inferior prognosis, and a crossresistant phenotype likely involves mechanisms distinct from single drug resistance, the current study focused on the genomics of multiple drug resistant ALL. To that end, we identified genes that are differentially expressed in ALL cells that exhibit de novo crossresistance to four widely used antileukemic agents and assessed their relationship to treatment outcome. Our results in 441 patients revealed two major components of de novo multiple drug resistance in childhood ALL, a common mechanism of crossresistance to all four drugs and an unanticipated mechanism of discordant resistance to VCR and ASP. Our findings also revealed a markedly inferior outcome of patients whose ALL cells exhibit a gene expression pattern indicative of these two forms of drug resistance (i.e., the worst CR and VCR-ASP score). Indeed, the treatment outcome of patients with both types of drug resistance (5 year DFS 37% ± 13%) is worse than the outcome observed in the worst subgroup previously identified by single drug resistance gene expression patterns (Figure S9; 5 year DFS 60% ± 9%) (Holleman et al., 2004). The aim of our prior study was to discover genes involved in single drug resistance, by classifying ALL cells as either sensitive or resistant to one of the four antileukemic agents (intermediates were excluded), and the combined gene expression score (sum of all four drugs) was predictive of treatment outcome. In the current study, the aim was to discover genes involved in multiple drug resistance using a different statistical method (PCA) on the LC_{50} values of four antileukemic agents in a much larger cohort of patients ($n = 441$). The first two principal components were used to find gene expression patterns significantly associated with these distinct patterns of multiple drug crossresistance. The 85% difference in genes identified in the two studies reflects the distinction between single drug and multiple drug

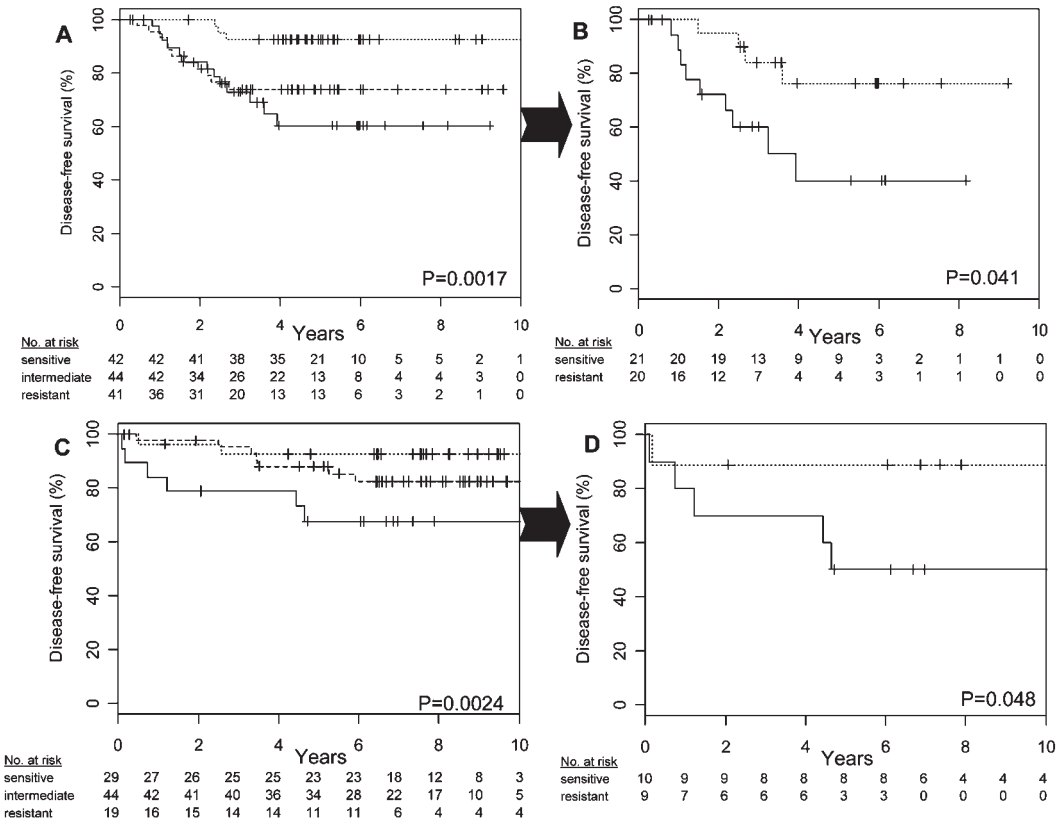

Figure 5. Crossresistance adds predictive value related to DFS to single drug resistance of PRD, VCR, ASP, DNR among patients with ALL

DFS using the COALL/DCOG cohort (n = 127) subdivided into equal groups (1/3) using the combined single PRD, VCR, ASP, DNR (PVAD) resistance score (**A**). After stratification by PVAD, the worst group (n = 41) was further divided into equal groups (1/2) by the multiple drug resistance gene expression (CR) score (**B**), revealing significant further discrimination by the multiple drug resistance gene expression pattern. Shown is the DFS in St. Jude patients (n = 92), divided by the single PVAD resistance score defined by the COALL/DCOG cohort (**C**). After stratification by PVAD gene expression score, the worst group (n = 19) was further subgrouped (1/2) by the multiple drug resistance (CR) gene expression score (**D**).

resistance. In further support of the distinct nature of our crossresistance gene pattern, the FDR is much lower, and the number of gene probe sets is much higher at different α (correlation p value cutoff), if the PCA based on the in vitro sensitivity data of all 441 patients is used compared to the PCA based on the in vitro sensitivity data of only 129 patients (Table S2). Finally, we found that resistance to mercaptopurine, an antileukemic agent not included in the analysis, was significantly related to the multiple drug crossresistance gene expression score, but there was no association with the combined single drug resistance gene expression score. This provides further evidence that the crossresistance phenotype reflects an intrinsic resistance mechanism that relates to multiple antileukemic drugs, beyond the four drugs we studied, and is distinct from our prior combined single drug resistance gene expression signature (sum of PVAD).

The present study identified 45 different genes and one cDNA clone (51 probe sets), the expression of which was significantly related to de novo crossresistance. The genes identified are involved in pathways that include transcription, transport, and cell cycle maintenance.

The biological and pharmacological relevance of genes discriminating crossresistant ALL is supported by their relationship to DFS in two different cohorts of patients. The 5 year DFS was only 53% in COALL/DCOG patients whose ALL cells exhibited a gene expression pattern indicative of crossresistance (top quartile by gene expression score), compared to 91% in patients with cross-sensitive gene expression scores (bottom quartile). The prognostic significance of genes discriminating crossresistance was confirmed in an independent cohort of patients treated with these drugs on a different protocol at St. Jude Children's Research Hospital. The 5 year DFS was only 56% in patients with ALL cells exhibiting a crossresistant gene expression pattern, compared to 100% in patients with a cross-sensitive gene expression pattern. The distinct nature of the crossresistance phenotype and gene expression pattern was further demonstrated by the ability of the CR gene expression pattern to further discriminate treatment outcome within the cohort of patients with the worst outcome as identified by the combined single drug resistance gene expression pattern (Holleman et al., 2004). This was evident to two independent cohorts of patients, treated on separate protocols in the United States and Europe. Moreover, the CR gene expression pattern was able to also discriminate de novo sensitivity to mechanistically distinct thiopurine antileukemic agents, whereas the combined single drug gene expression score (PVAD) was not.

In addition to the crossresistance phenotype, our PCA iden-

tified a VCR-ASP discordant resistance phenotype as the second strongest component of multiple drug resistance. We found 139 unique known genes and 13 cDNA clones (200 probe sets), the expression of which were significantly related to VCR-ASP discordant resistance. Patients whose ALL cells exhibited a pattern of gene expression associated with VCR sensitivity and ASP resistance were predominately B lineage ALL that were not hyperdiploid (>50 chromosomes) and did not carry the *TEL-AML1* gene fusion. In contrast, patients whose ALL cells were VCR resistant and ASP sensitive were commonly either *TEL-AML1* positive or hyperdiploid, providing insights into why these genetic subtypes of ALL have a favorable prognosis, in particular their greater sensitivity to ASP (Kaspers et al., 1995; Ramakers-van Woerden et al., 2000; Stams et al., 2003).

Seventy-five of the 200 gene probe sets (38%) discriminating the VCR-ASP discordant sensitivity phenotype encode ribosomal proteins. We have previously shown that overexpression of ribosomal genes is associated with ASP resistance (Holleman et al., 2004), but this provides evidence that overexpression of the same ribosomal protein genes is associated with increased VCR sensitivity. ASP has been shown to selectively suppress synthesis of ribosomal proteins at the level of mRNA translation and targets a common p70 S6K kinase/eukaryotic initiation factor 4E binding protein 1 signaling pathway in leukemia cells (Iiboshi et al., 1999). ASP has also been shown to block protein synthesis and to inhibit synthesis of rRNA (Nakashima et al., 1976; Story et al., 1993). Overexpression of protein synthesis genes that are downstream of ASP effects may therefore protect cells or delay ASP toxicity. Our findings show that overexpression of ribosomal protein genes has an opposite effect on VCR resistance, and this phenotype is characteristic of a novel ALL subgroup. Our findings also suggest that, if ribosomal protein inhibitors reverse ASP resistance, they may have an opposite effect on VCR sensitivity.

Resistance to econazole and crossresistance to endoplasmic reticulum stress-inducing drugs in HL60 cells can be partially reversed by the ribosome-inactivating protein saporin (Zhang and Berger, 2003), but such strategies have yet to be tested for reversing ASP resistance. With contemporary ALL treatment regimens, ASP dose intensity appears to be an important determinant of treatment response (Silverman et al., 2001; Silverman and Sallan, 2003). Furthermore, in vivo ASP resistance is associated with poor treatment outcome (Asselin et al., 1999). Taken together with our current findings, the administration of ribosomal protein inhibitors may be a viable approach to overcome ASP resistance, but such strategies would need to be designed to avoid offsetting effects on VCR sensitivity.

The expression of genes discriminating VCR-ASP discordant resistance was significantly related to treatment outcome in patients treated on the COALL/DCOG protocols. In these patients, those with ALL classified as ASP resistant and VCR sensitive had a 5 year DFS of 56%, compared to 93% in patients whose ALL cells were ASP sensitive and VCR resistant. Interestingly, the VCR-ASP gene expression pattern was not of prognostic significance in patients treated on the St. Jude protocol. Why the VCR-ASP discordant phenotype was significantly related to treatment outcome in COALL/DCOG protocols but not the St. Jude treatment cannot be explained by differences in total cumulative dose of ASP. However, the schedule of ASP administration was different in these protocols, with the St. Jude protocol administering ASP 12 to 25 times compared to 5 to 6 times in the COALL and 4 to 13 times in the DCOG protocols (Harms and Janka-Schaub, 2000; Harms et al., 2003; Pui et al., 2000; Pui et al., 2004b).

The COALL and DCOG protocols also comprise less overall intensive therapy than the St. Jude protocol. Etoposide was extensively used in the St. Jude protocol (total of four doses of 300 mg/m^2 during induction and postremission therapy, plus HR consolidation therapy), whereas etoposide was not used in COALL/DCOG. In the COALL protocol, the mechanically similar drug teniposide (165 mg/m^2) was given, but only one dose was given for low-risk patients, and two doses were given for high-risk patients. Additionally, cytarabine in the St. Jude protocols was given in higher doses and for an extended time period compared to COALL and DCOG. Thus, the COALL/DCOG protocols rely more heavily on ASP, and consequently treatment outcome may be more strongly influenced by the VCR-ASP phenotype.

It is not known whether the expression of genes associated with the VCR-ASP discordant resistance phenotypes is caused by the *TEL-AML1* gene fusion or hyperdiploidy and is the basis for their favorable prognosis. Likewise, it is not known whether overexpression of ribosomal proteins contributes to the favorable prognosis of these ALL subtypes when treated on ASP-intensive protocols (Pui and Evans, 1998; Zaza et al., 2004). After the VCR-ASP score was adjusted for ALL subtype, the majority of significant genes were still linked to protein synthesis. Our current findings have identified drug resistance phenotypes and gene expression patterns that provide insights into why these ALL subtypes have a better outcome.

In the current study, we only analyzed B lineage ALL patients, because T lineage ALL cells have a distinct gene expression profile, and there were too few T-ALL cases for an independent analysis (Ross et al., 2003; Yeoh et al., 2002; Holleman et al., 2004). T lineage ALL is generally more resistant to PRD, ASP, DNR, and VCR in vitro (Pieters et al., 1998), and the VCR-ASP discordant resistant gene expression score was significantly higher for this subset of patients in the current study (data not shown). Because T-ALL has a worse prognosis with many treatment protocols and may have distinct drug resistance mechanisms, it will be important to extend the current studies to this major ALL subtype.

In summary, the current work has revealed genes that are expressed at significantly different levels in ALL cells that exhibit crossresistance to four widely used antileukemic agents and has shown that the expression pattern of these genes in ALL cells is significantly related to treatment response and is able to identify a subgroup with an inferior outcome when compared to gene expression patterns associated with single drug resistance. Furthermore, the current studies have identified a phenotype of discordant sensitivity to ASP and VCR and revealed a gene expression signature comprising a large number of ribosomal protein genes that discriminates ALL cells exhibiting discordant sensitivity to these two widely used antileukemic agents. Together, these findings provide insights into de novo crossresistance of ALL and point to potential strategies to circumvent these mechanisms of treatment failure.

Experimental procedures

Leukemia samples

Leukemic cells were isolated from bone marrow aspirates or peripheral blood from 441 patients with diagnosed B lineage ALL, enrolled on the

DCOG ALL-9 protocol in the Sophia Children's Hospital, or the COALL 92 or 97 protocol. In vitro cytotoxicity was determined for PRD, VCR, ASP, and DNR utilizing the 4 day in vitro MTT drug resistance assay, as we have previously described (den Boer et al., 2003; Kaspers et al., 1997; Pieters et al., 1990). For each patient, we determined the lethal concentration to 50% of leukemia cells (LC_{50}) for each of the four drugs. ALL cells isolated from bone marrow or peripheral blood samples of a subset of these patients (n = 129) were also analyzed for gene expression, as described below. 147 of the 441 patients in the LC_{50} analysis and 127 of the 129 patients in the gene expression analysis were part of a prior study of single drug resistance (Holleman et al., 2004). The ALL subtypes (i.e., >50 chromosomes, *TEL-AML1*, *MLL-AF4*, *BCR-ABL*, and *E2A-PBX1*) for the 129 patients in gene expression subset were determined by flow cytometry, PCR, or FISH, as previously described (Pui and Evans, 1998).

A separate cohort of 83 B lineage ALL patients, treated on the St. Jude Children's Research Hospital Total Therapy 13A and 13B protocols, served as an independent "test set" for the drug resistance gene expression model and outcome analyses (Ross et al., 2003; Yeoh et al., 2002). Gene expression in ALL cells was previously reported for all 83 patients (Ross et al., 2003; Yeoh et al., 2002), and these patients are among the independent test set for single drug resistance (Holleman et al., 2004). Mercaptopurine in vitro resistance was determined at St. Jude in 51 patients enrolled on Total Therapy 15. All patients or their parents (or guardians) provided informed consent, and local institutional review boards approved all protocols.

Oligonucleotide microarray analysis

Total RNA from leukemia cells of 129 patients treated on the COALL/DCOG protocols was hybridized to the U133A GeneChip oligonucleotide microarrays containing 22,284 probe sets (~12,700 genes), according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Gene expression signals were scaled to the target intensity of 2500, using Affymetrix Microarray Suite (MAS) 5.0 software. Probe sets expressed in less than three percent of the patients were excluded, leaving 14,550 probe sets for subsequent analyses. For illustration of the PCA and the hierarchical clustering, we used GeneMaths 2.1 software (Applied Maths, St. Martens-Latem, Belgium).

Statistical analyses

LC_{50} values were first standardized within each drug across all 441 patients to have the mean equal to zero and one standard deviation equal to one. This permitted analyses across drugs with equal weighting. PCA was applied to reduce the dimensionality of multivariate data (LC_{50} values for all four drugs in all 441 patients) by transforming the original variables into uncorrelated new ones (the principal components) that account for decreasing proportions of variance. Two scores, crossresistance to all drugs (CR score) and discordant resistance to VCR and ASP (VA score) were assigned to each patient, corresponding to the first two (most important) components of the PCA.

$$\text{CR score} = 0.544{*}LC_{50}\text{PRD} + 0.428{*}LC_{50}\text{VCR} + 0.394{*}LC_{50}\text{ASP} + 0.604{*}LC_{50}\text{DNR}$$

$$\text{VA score} = -0.121{*}LC_{50}\text{PRD} + 0.683{*}LC_{50}\text{VCR} - 0.715{*}LC_{50}\text{ASP}$$

To adjust for ALL subtype, a linear model was fitted for the second component of the PCA with genetic subtype, as the covariate and the residues obtained from the linear model were used as the adjusted VCR-ASP score. Spearman's rank correlation was applied to assess the relation between the CR score or VCR-ASP score and the log-transformed gene expression values of each probe set. The Spearman's rank correlation (rho) is commonly used as the estimate for the association between two continuous variables and is similar to R^2. Probe sets were ranked according to p values, as computed by the Spearman's rank correlation. Selection of genes associated with the CR and VCR-ASP score was guided by the FDR (Storey and Tibshirani, 2003) and crossvalidation (10-fold). In each of ten iterations of crossvalidation, a tenth of the samples were left out, and scores were predicted based on the other 90% of the samples; the observed and the predicted score were highly correlated and resulted in a high rho, indicating the predictor was robust. For each patient, the gene expression score was defined as the weighted average of the expression signal of the top selected genes, with the weight being the correlation coefficient determined between the expression of each of the selected genes and the CR or the VCR-ASP score in 129 children with B lineage ALL.

We determined the over- or underrepresentation of discriminating genes in functional groups compared to the human genome as represented by all genes on the U133A chip, using Fisher's exact test and information provided by the Gene Ontology Consortium (http://www.geneontology.org/) and Affymetrix NetAffx (https://www.affymetrix.com/). Through NCBI's Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/, primary data are accessible (Platform GPL 96; Samples GSM 9653-8, 9694-6, 9698-9, 9701-4, 9707-20, 9723-29, 9733-39, 9741, 9743-51, 9753-55, 9757-62, 9765-7, 9769-73, 9775, 9777-88, 9790-1, 9794-7, 9799, 9801, 9803-4, 9807-12, 9814-19, 9821, 9824-6, 9828-9, 9831, 9833-4, 9836, 9840, 9842,9845-6, 9848-9, 9851, 9862, 9564, 9932-4, 44303-4; Series GSE2351), and additional information is provided in the Supplemental Data and at http://www.stjuderesearch.org/data/ALL5/ or http://www.eur.nl/fgg/kgk/.

Survival analysis methods were used to estimate the probability of DFS in crossresistant (top quartile), intermediate (middle two quartiles), or crosssensitive (bottom quartile) patients, according to the CR gene expression score defined by the first component of the PCA. Likewise, DFS was compared for patients classified as VCR sensitive plus ASP resistant (top quartile), VCR resistant plus ASP sensitive (bottom quartile), and all others, according to the VCR-ASP gene expression score defined by the second component. The duration of DFS was defined as the time from diagnosis until the date of leukemia relapse, where any type of leukemia relapse was considered an event. Second malignancies and death due to other reasons were censored at the time of occurrence. Time was also censored at the last follow-up date if no failure was observed. Cox proportional hazards regression analysis, as modified by Fine and Gray (Fine and Gray, 1999), was used to assess the association of the CR and VCR-ASP gene expression score with DFS.

Supplemental data

The Supplemental Data include eleven supplemental tables and nine supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/7/4/375/DC1/.

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