Drug–Gene Modeling in Pediatric T-Cell Acute Lymphoblastic Leukemia Highlights Importance of 6-Mercaptopurine for Outcome

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Abstract

Patients relapsing with T-cell acute lymphoblastic leukemia (T-ALL) face a dismal outcome. The aim of this study was to identify new markers of drug resistance and clinical response in T-ALL. We measured gene expression and drug sensitivity in 15 pediatric T-ALL cell lines to find signatures predictive of resistance to 10 agents used in therapy. These were used to generate a model for outcome prediction in patient cohorts using microarray data from diagnosis specimens. In three independent T-ALL cohorts, the 10-drug model was able to accurately identify patient outcome, indicating that the in vitro–derived drug–gene profiles were clinically relevant. Importantly, predictions of outcome within each cohort were linked to distinct drugs, suggesting that different mechanisms contribute to relapse. Sulfite oxidase (SUOX) expression and the drug-transporter ABCC1 (MRP1) were linked to thiopurine sensitivity, suggesting novel pathways for targeting resistance. This study advances our understanding of drug resistance in T-ALL and provides new markers for patient stratification. The results suggest potential benefit from the earlier use of 6-mercaptopurine in T-ALL therapy or the development of adjuvants that may sensitize blasts to this drug. The methodology developed in this study could be applied to other cancers to achieve patient stratification at the time of diagnosis. Cancer Res; 73(9); 2749–59. ©2013 AACR.

Introduction

Improved survival for pediatric acute lymphoblastic leukemia (ALL) patients is one of the success stories of cancer research. From an essentially fatal disease 50 years ago, the empirical development of complex multiagent chemotherapy has resulted in the achievement of overall 5-year survival rates of ~90% (1, 2). However, lower 5-year survival rates are seen in infants (<1 year of age, 53%) and those with T-lineage ALL (T-ALL, 81%). For the significant number of patients that experience relapse, survival rates are dramatically reduced and worsen with each subsequent disease recurrence (3). This adverse trend is even more pronounced in T-ALL and those with known clinical risk factors (3). To further improve outcomes, many researchers have attempted to develop models that can be used to predict clinical outcome based on gene-expression patterns present in patients’ leukemia cells at the time of diagnosis (4–12). Comparison of these profiles with those observed at relapse has yielded important clues to the differences that emerge following therapy failure (13–15). An alternative but related approach has been to compare diagnostic gene-expression profiles between patients with demonstrated differences in clinical response to remission induction therapy or in their sensitivity to these agents as measured ex vivo (16–19). However, the influence of other agents, such as those used in consolidation and maintenance therapy have not been studied in this way.

To address this we have interrogated an in-house dataset of in vitro–derived resistance and gene expression profiles to generate a model that can predict drug sensitivity and therefore outcome, incorporating data from all 10 chemotherapy agents commonly used in T-ALL therapy. In addition to revealing insights into the pathways associated with drug resistance to each of the 10 different drugs, we have been able to use this drug–gene model to accurately predict outcome in 3 independent cohorts of T-ALL patients. This novel approach revealed that relapse in individual patients is linked to distinct drugs, and highlights a particular importance for 6-mercaptopurine (6MP) in determining outcome in T-ALL. Based on these findings, we suggest that there may be a role for the use of 6MP in earlier phases of T-ALL therapy.

Materials and Methods

Cell lines and drug-sensitivity profiling

The cell line panel used in this study has been previously described and comprises 9 T-ALL lines derived in our own laboratory from pediatric ALL bone marrow specimens (PER cell lines), plus 6 additional T-ALL cell lines obtained from...
external sources (20, 21). Cell lines were grown in RPMI-1640 supplemented with 2 mmol/L l-glutamine, 10 mmol/L 2-mercaptoethanol, and 10% to 20% heat-inactivated fetal calf serum. The media for PER-cell lines contained additional nonessential amino acids and pyruvate, whereas 300 units/mL interleukin-2 is required for growth of PER-427 and PER-487. The sensitivity of the T-ALL cell lines to methylprednisolone (MPRED), dexamethasone (DEX), cytosine arabinoside (ARA-C), 6-thioguanine (6TG), 6-mercaptopurine (6MP), daunorubicin (DNR), doxorubicin (DOX), l-asparaginase (ASP), vincristine (VCR), and methotrexate (MTX) has been previously published (20) and was measured using the MTT assay with drugs incubated for 4 days. The IC_{50} (drug concentration that inhibits cell growth by 50%) was used as the measure of drug response, with the IC_{50} being recorded as double the highest concentration tested in cases where 50% cytotoxicity could not be achieved (20). DNA fingerprinting confirmed the identity of each of the cell lines (20).

**Patient cohorts**

Gene expression profiling of the primary patient cohort analyzed in this study has been previously described (7) and comprised 50 T-ALL patients treated on Children's Oncology Group (CCG/COG) protocol 1961 for high-risk ALL (22). Bone marrow specimens were obtained at diagnosis from patients at either the Princess Margaret Hospital, Perth, Australia or provided by COG. Ethical approval was obtained from the Institutional Review Board, and informed consent for the use of tissues was obtained for all individuals. All patients achieved remission following induction therapy; those patients achieving complete continuous remission (CCR) had median follow-up times of 7.3 years, whereas 44% of the patients subsequently relapsed (R). Expression data from the 2 Winter and colleagues cohorts (microarray CEL files and patient details) were obtained from the authors (23) and normalized by robust multiarray analysis (RMA) as previously described (7). Induction failure cases were removed from these cohorts before analysis, resulting in cohort sizes of 44 patients for Pediatric Oncology Group (POG/COG) Protocol 9404 (30 CCR, 14R, measured on HG-U133 Plus 2.0 arrays) and 41 patients for POG-8704 (24 CCR, 17R, measured on HG-U133A arrays).

**Gene expression profiling**

Briefly, RNA was extracted from cell lines in exponential growth phase and hybridized to Affymetrix HG-U133A microarrays (ArrayExpress Accession E-MTAB-1208; refs. 11 and 13). RNA from the CCG-1961 T-ALL cohort (n = 50) was extracted from bone marrow specimens and hybridized to HG-U133Plus 2.0 GeneChips (ArrayExpress Accession E-MTAB-1205; ref. 7). Microarray data were normalized using RMA as previously described (5, 13). Gene expression values across the T-ALL cell line panel (n = 15 cell lines) were log_{2} transformed and correlated against log_{2} IC_{50} scores from the same panel using Pearson's correlation as described (11) to generate individual lists of genes correlating with the sensitivity profile for each drug (n = 10 drugs). Drug pairs belonging to the same drug class, namely MPRED and DEX (glucocorticoids), DOX and DNR (anthracyclines), and 6MP and 6TG (thiopurines), were cross-referenced to identify genes most significantly correlated against both agents in each class. Genes with significant correlation scores against these 3 drug pairs and the 4 additional single agents were interrogated for biological relevance using Ingenuity Pathway Analysis (Ingenuity Systems Inc.) to identify pathways associated with resistance. To generate a global gene expression–phenotype profile for all drugs, the top 50 correlating probe sets for each individual drug were combined (resulting in 380 nonredundant probe sets) and similarly analyzed using Ingenuity Pathway Analysis.

**Ten drug modeling for prediction of clinical outcome**

Modeling of in vitro–derived drug–gene data was done in 2 stages to derive testable predictions of prognosis in patient cohorts. First, for individual drugs, we conducted principal component analysis (PCA) using log_{2} gene expression values from the top 50 probe sets correlating with drug IC_{50} within the T-ALL cell line panel. The top 10 principal components from each PCA were then used as covariates in linear regression analysis to generate models for the prediction of IC_{50} values (resistance scores) in the cell line data for each drug. The use of PCA and linear regression of the obtained components was designed to collapse the larger number of variables (50 probe sets/drug) for modeling within the smaller number of cell lines (n = 15). Second, these in vitro–derived models of drug resistance were applied to gene-expression datasets from 3 different T-ALL patient cohorts (see Materials and Methods) to predict in vivo correlates of resistance that could be used to stratify patients into those with a low or high probability of relapse. To do this, the PCA rotation matrix for each of the 10 drugs developed using the cell line data was directly applied to the expression data from each patient cohort, and the resulting principal component scores were used in the linear regression models previously developed for each drug to predict “resistance scores” in individual patients. We then used these “resistance scores” in logistic regression to model the probability of relapse for each patient. This probability was converted into a prediction of CCR/relapse labels using a probability cut-off point of 50%, generating model accuracies, sensitivity (the percentage of correctly predicted relapse patients), specificity (the percentage of correctly predicted nonrelapse patients), positive predictive values (the proportion of patients among those predicted to relapse that actually relapsed), and negative predictive values (the proportion of patients among those predicted as nonrelapse that actually achieved CCR). This stratification was also used for Kaplan–Meier survival analysis, with significance determined by log-rank test. The logistic regression models used to predict outcome in each patient cohort based on the calculated multiple-drug resistance scores are provided in Supplementary Table S5.

**Drug contribution analysis**

To assess whether, for any given patient, a prediction of relapse within our model might be driven by the influence of one or several drugs in particular, we first scored the positive or negative influence of each drug within the model (predicted resistance score × equation coefficient), and summed the total.

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positive and negative scores for all drugs for each patient. The balance of these scores (plus equation constant) determines the prediction of outcome, with positive sums being associated with relapse, and negative values associated with nonrelapse. In real-world terms this represents the balance of resistances (positive effects) and sensitivities (negative effects) to the multiple drugs used to treat patients, the outcome of which contributes to a patient’s prognosis. For each patient for whom we were able to correctly predict outcome (relapse/nonrelapse), the contribution of each drug was therefore calculated as a percentage of the averaged total score within the cohort required to be reached to definitively generate that prediction. This generates an estimate of the relative contribution for each drug toward the correct prediction of outcome for each patient in that particular cohort.

To assess whether there might be groups of patients sharing similar drug contribution profiles, we conducted unsupervised hierarchical clustering using these calculated drug contribution scores and a correlative distance metric (1 – r). This identified subpopulations of patients for whom the pattern of drug influence on outcome was similar. Finally, we calculated the frequency with which particular drugs were found to be the strongest drivers for the accurate prediction of outcome in each cohort by counting all occurrences where a drug had either the highest contribution score for a particular patient, or a contribution score greater than 50% of the score for most prominent drug for that patient.

For each patient for whom that drug (or drug-class) was a major determinant for the accurate prediction of outcome (relapse/nonrelapse), the contribution of each drug was therefore calculated as a percentage of the averaged total score within the cohort required to be reached to definitively generate that prediction. This generates an estimate of the relative contribution for each drug toward the correct prediction of outcome for each patient in that particular cohort.

Table 1. Top genes correlated to glucocorticoids and ASP resistance in T-ALL cell lines [correlation coefficients vs. IC\textsubscript{50} (all \(P < 0.0005\))]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Title</th>
<th>MPRED</th>
<th>DEX</th>
<th>Gene</th>
<th>Title</th>
<th>L-Asparaginase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX14</td>
<td>SRY (sex determining region Y)-box 14</td>
<td>0.91</td>
<td>0.88</td>
<td>NRP1L3</td>
<td>Nitrogen permease regulator-like 3</td>
<td>0.93</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed-lineage leukemia gene</td>
<td>−0.90</td>
<td>−0.86</td>
<td>CREB3</td>
<td>cAMP responsive element binding protein</td>
<td>0.92</td>
</tr>
<tr>
<td>NMT2</td>
<td>N-myristoyltransferase 2</td>
<td>−0.87</td>
<td>−0.87</td>
<td>AV719355</td>
<td>Unknown</td>
<td>−0.90</td>
</tr>
<tr>
<td>NM_025012</td>
<td>Unknown</td>
<td>−0.87</td>
<td>−0.84</td>
<td>SMC3</td>
<td>Structural maintenance of chromosomes 3</td>
<td>−0.88</td>
</tr>
<tr>
<td>CCNL1</td>
<td>Cyclin L1</td>
<td>−0.87</td>
<td>−0.81</td>
<td>KIAA1109</td>
<td>KIAA1109</td>
<td>−0.88</td>
</tr>
<tr>
<td>SLC35B1</td>
<td>Solute carrier family 35, member B1</td>
<td>0.86</td>
<td>0.86</td>
<td>PCBP2</td>
<td>Poly(rC) binding protein 2</td>
<td>−0.88</td>
</tr>
<tr>
<td>PSMC5</td>
<td>Proteasome 26S subunit, ATPase, 5</td>
<td>0.86</td>
<td>0.84</td>
<td>APBA3</td>
<td>Amyloid β (A4) precursor binding protein</td>
<td>0.88</td>
</tr>
<tr>
<td>MED24</td>
<td>Mediator complex subunit 24</td>
<td>0.85</td>
<td>0.81</td>
<td>LDLRAP1</td>
<td>Low-density lipoprotein receptor adaptor 1</td>
<td>0.87</td>
</tr>
<tr>
<td>CLTC</td>
<td>Clathrin, heavy chain (Hc)</td>
<td>0.84</td>
<td>0.88</td>
<td>PSAT1</td>
<td>Phosphoserine aminotransferase 1</td>
<td>0.87</td>
</tr>
<tr>
<td>POLI</td>
<td>Polymerase (DNA directed) iota</td>
<td>−0.84</td>
<td>−0.86</td>
<td>HELL5</td>
<td>Helicase, lymphoid-specific</td>
<td>−0.86</td>
</tr>
<tr>
<td>PEX14</td>
<td>Peroxisomal biogenesis factor 14</td>
<td>0.83</td>
<td>0.86</td>
<td>MED27</td>
<td>Mediator complex subunit 27</td>
<td>0.86</td>
</tr>
<tr>
<td>NDUFB1</td>
<td>NADH dehydrogenase (ubiquinone) 1β</td>
<td>−0.82</td>
<td>−0.85</td>
<td>UPF3A</td>
<td>UPF3 regulator of nonsense transcripts</td>
<td>−0.86</td>
</tr>
</tbody>
</table>

**Results**

**Genes and pathways associated with drug resistance in T-ALL cell lines**

Our laboratory has developed an authenticated panel of pediatric T-ALL cell lines that have been grown in the absence of drug selection. These cultures retain critical features of the primary disease and their drug-resistance profiles parallel the spectrum of resistance that has been observed in primary patient specimens (20). We have previously examined the baseline resistance of these 15 T-ALL cell lines to the glucocorticoids DEX and MPRED (20) and correlated the data with gene expression profiles as determined by HG-U133A microarray (11). Here we have extended this study to examine the drug-resistance profiles of 8 additional drugs used for pediatric ALL. To reduce noise and increase the power of the analysis, data from drugs belonging to the same drug class (i.e., anthracyclines, glucocorticoids, and thiopurines) were cross-referenced to produce gene correlates for the drug class rather than the individual agents.

The top correlating genes for each of the drug pairs and the 4 additional individual drugs are shown in Tables 1 to 4, whereas the biological processes associated with these gene sets (Ingenuity Pathway Analysis) are shown in Fig. 1A (further details provided in Supplementary Table S3). Pathways involved in cancer signaling, cell death, growth and proliferation, and metabolism were strongly associated with most drug classes (Fig. 1A). The biological features revealing the most significant associations in this analysis were for thiopurines (gene expression, differentiation/development, cell growth and proliferation, and cell death), anthracyclines (cancer signaling...
pathways), and ASP (posttranslational modification and metabolism). In studying these drug–gene profiles it quickly became apparent that there was a strong reciprocity in the relationship between glucocorticoids and MTX. This is showed in Fig. 1B, which shows that the most significantly correlated probe sets for MPRED were significantly inversely correlated with MTX. To interpret the biological significance of this observation we generated a glucocorticoid–MTX reciprocity index by identifying all probe sets with a significant correlation coefficient for both MPRED and DEX that were significantly, but oppositely, correlated with MTX resistance (Pearson correlation, \( P < 0.05 \) for each). The glucocorticoid–MTX reciprocity index (labeled “GC.MTX” in Fig. 1A) returned one of the broadest biological phenotypes with Ingenuity Pathway Analysis, but several pathways appeared that were not associated with the glucocorticoid and MTX signatures individually (e.g.,

### Table 2. Top genes correlated to thiopurines and MTX resistance in T-ALL cell lines [correlation coefficients vs. \( IC_{50} \) (all \( P < 0.005 \)]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Title</th>
<th>6MP</th>
<th>6TG</th>
<th>Gene</th>
<th>Title</th>
<th>MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUOX</td>
<td>Sulfite oxidase</td>
<td>0.79</td>
<td>0.92</td>
<td>CD200</td>
<td>CD200 molecule</td>
<td>0.92</td>
</tr>
<tr>
<td>GTPBP10</td>
<td>GTP-binding protein 10 (putative)</td>
<td>−0.86</td>
<td>−0.74</td>
<td>HRSP12</td>
<td>Heat-responsive protein 12</td>
<td>−0.90</td>
</tr>
<tr>
<td>MON1B</td>
<td>MON1 homolog B (yeast)</td>
<td>0.83</td>
<td>0.76</td>
<td>MPI</td>
<td>Mannose phosphate isomerase</td>
<td>−0.90</td>
</tr>
<tr>
<td>SLC25A16</td>
<td>Mitochondrial solute carrier (Graves disease)</td>
<td>0.80</td>
<td>0.79</td>
<td>SUCLG2</td>
<td>Succinate-CoA ligase, GDP-forming, ( \beta )</td>
<td>−0.88</td>
</tr>
<tr>
<td>ACADSB</td>
<td>Acyl-coenzyme A dehydrogenase</td>
<td>0.84</td>
<td>0.73</td>
<td>ATP2B4</td>
<td>ATPase, Ca( ^{2+} ) transporting, plasma membrane 4</td>
<td>0.86</td>
</tr>
<tr>
<td>NM_018687</td>
<td>Hepatocellular carcinoma-assoc. gene TD26</td>
<td>−0.76</td>
<td>−0.79</td>
<td>HDDD2</td>
<td>HD domain containing 2</td>
<td>−0.85</td>
</tr>
<tr>
<td>AL023773</td>
<td>Similar to melanoma antigen family C2</td>
<td>−0.77</td>
<td>−0.77</td>
<td>VPS37C</td>
<td>Vacuolar protein sorting 37 homolog C</td>
<td>−0.84</td>
</tr>
<tr>
<td>J00146</td>
<td>Dihydropolate reductase pseudogene</td>
<td>−0.81</td>
<td>−0.73</td>
<td>PDLA5</td>
<td>Protein disulfide isomerase family A5</td>
<td>−0.84</td>
</tr>
<tr>
<td>ABCC1</td>
<td>Multidrug resistance protein 1</td>
<td>0.73</td>
<td>0.79</td>
<td>SCRIN3</td>
<td>Secerin 3</td>
<td>0.84</td>
</tr>
<tr>
<td>GPR161</td>
<td>G protein–coupled receptor 161</td>
<td>−0.75</td>
<td>−0.77</td>
<td>MRPS3S</td>
<td>Mitochondrial ribosomal protein S33</td>
<td>−0.84</td>
</tr>
<tr>
<td>C19orf54</td>
<td>Chromosome 19 open reading frame 54</td>
<td>−0.74</td>
<td>−0.76</td>
<td>CDK3RAP3</td>
<td>CDK5 regulatory subunit associated protein 3</td>
<td>0.84</td>
</tr>
<tr>
<td>WIPF1</td>
<td>WAS/WASL interacting protein family, 1</td>
<td>0.73</td>
<td>0.75</td>
<td>PPM1A</td>
<td>Protein phosphatase 1A (formerly 2C, alpha)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

### Table 3. Top genes correlated to anthracyclines and ARAC resistance in T-ALL cell lines [correlation coefficients vs. \( IC_{50} \) (all \( P < 0.005 \)]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Title</th>
<th>DOX</th>
<th>DNR</th>
<th>Gene</th>
<th>Title</th>
<th>ARAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMK2G</td>
<td>Ca( ^{2+} )/calmodulin-dependent protein kinase I( \gamma )</td>
<td>0.77</td>
<td>0.84</td>
<td>ACP2</td>
<td>Acid phosphatase 2, lysosomal</td>
<td>0.88</td>
</tr>
<tr>
<td>TKTL1</td>
<td>Transketolase-like 1</td>
<td>0.73</td>
<td>0.88</td>
<td>TRAF3</td>
<td>TNF receptor-associated factor 3</td>
<td>0.85</td>
</tr>
<tr>
<td>GRAP2</td>
<td>GRB2-related adaptor protein 2</td>
<td>−0.75</td>
<td>−0.86</td>
<td>TBCD</td>
<td>Tubulin folding cofactor C</td>
<td>0.84</td>
</tr>
<tr>
<td>UBE2D2</td>
<td>Ubiquitin-conjugating enzyme E2D 2</td>
<td>−0.74</td>
<td>−0.87</td>
<td>MFSD10</td>
<td>Major facilitator superfamily domain 10</td>
<td>0.82</td>
</tr>
<tr>
<td>ASAP3</td>
<td>ArfGAP with SH3, ankyrin repeat</td>
<td>−0.80</td>
<td>−0.77</td>
<td>CSPC1</td>
<td>Centrosome/spindle pole associated protein 1</td>
<td>−0.82</td>
</tr>
<tr>
<td>SSR3</td>
<td>Signal sequence receptor, ( \gamma )</td>
<td>0.75</td>
<td>0.81</td>
<td>PPAP2B</td>
<td>Phosphatic acid phosphatase type 2B</td>
<td>−0.82</td>
</tr>
<tr>
<td>SPTAN1</td>
<td>Spectrin, ( \alpha ), nonerythrocytic 1</td>
<td>−0.80</td>
<td>−0.77</td>
<td>YIPF6</td>
<td>Yip1 domain family, member 6</td>
<td>0.81</td>
</tr>
<tr>
<td>TRPC3</td>
<td>Transient receptor potential cation channel C3</td>
<td>−0.82</td>
<td>−0.74</td>
<td>GNAO1</td>
<td>Guanine nucleotide binding protein, ( \alpha ) activity</td>
<td>−0.81</td>
</tr>
<tr>
<td>ZNF692</td>
<td>Zinc finger protein 692</td>
<td>0.74</td>
<td>0.79</td>
<td>EIF1AY</td>
<td>Eukaryotic translation initiation factor 1A, Y</td>
<td>−0.80</td>
</tr>
<tr>
<td>APH1A</td>
<td>Anterior pharynx defective 1 homolog A</td>
<td>0.73</td>
<td>0.78</td>
<td>AK024177</td>
<td>Unknown</td>
<td>0.80</td>
</tr>
<tr>
<td>KIAA0885</td>
<td>KIAA0885</td>
<td>−0.75</td>
<td>−0.74</td>
<td>RAB6B</td>
<td>RAB6B, member RAS oncogene family</td>
<td>−0.79</td>
</tr>
<tr>
<td>DLG3</td>
<td>Discs, large homolog 3</td>
<td>−0.73</td>
<td>−0.76</td>
<td>MED16</td>
<td>Mediator complex subunit 16</td>
<td>0.78</td>
</tr>
</tbody>
</table>
HIF-signaling, ER-signaling, and posttranslational modification). This reciprocity is an unexpected relationship that we have reported previously (11, 20) and although the mechanism is not clear, the relevance for the clinic is that cross-resistance, which is often observed between drugs in ALL, is not expected to develop between glucocorticoids and MTX. This lack of cross-resistance is supported by the findings of a small drug-sensitivity study in primary ALL specimens published more than a decade ago (24). Moreover, our data indicate that the transcriptional networks that underpin a resistant phenotype for one drug class may actually sensitize cells to the other, that is, in patients with demonstrated resistance to glucocorticoids for example, MTX should not only continue to be effective but its efficacy may in fact be increased. A more detailed breakdown of the gene networks uniquely represented or enhanced within the GC.MTX signature (Supplementary Table S3), shows that protein synthesis, particularly eukaryotic initiation factor 2 (eIF2) signaling, is the most prominent feature emerging from this gene set ($P = 2.17 \times 10^{-7}$), suggesting that control of translation is one of the critical pathways determining the relative balance of glucocorticoid and MTX sensitivity within the cell. The emergence of estrogen-receptor signaling within the GC.MTX signature (Fig. 1A; Supplementary Table S3), which is clearly highly relevant for glucocorticoid resistance, may be explained by such changes to translational control. Finally, it has been reported that the activity of ABCG2, one of the major drug transporters for MTX, is inhibited by DEX and MPRED (25), and although there was no significant correlation of ABCG2 expression with resistance to these drugs in this study, it remains possible that posttranscriptional effects on ABCG2 of MPRED (25), and although there was no significant correlation of ABCG2 expression with resistance to these drugs in this study, it remains possible that posttranscriptional effects on ABCG2 or other drug transporters also contribute to the observed GC.MTX reciprocity.

Among the top-ranked genes correlating with resistance to the agents in Fig. 1, there were several of particular biological interest. Highly negatively correlated with glucocorticoids was the mixed lineage leukemia (MLL) gene (Table 1), the expression of which we have previously showed to be associated with this drug-class in T-ALL (10, 11). RNAi knockdown of MLL expression in T-ALL cell lines significantly increased resistance to DEX and γ irradiation indicating an important role for wild-type MLL in the control of cellular apoptosis (10). The top gene correlated with ASP resistance (Table 1) was Nitrogen Permease Regulator-Like 3 (NPRL3), thought to be part of a complex that mediates autophagy (26) and amino acid starvation signals to TORCI, the mTOR complex responsible for monitoring cellular nutrient status (27). This is highly relevant given the action of ASP in depleting asparagine levels. Asparagine synthetase (ASN5), the upregulation of which is a known mechanism of ASP resistance, was also significantly correlated with ASP resistance in T-ALL cell lines (Supplementary Table S2).

Genes known to alter the cellular sensitivity to thiopurines include the phase II enzyme thiopurine S-methyltransferase (TPMT) and the cellular oxidoreductase xanthine oxidase (28, 29). No correlation between the expression of these genes and thiopurine IC50 was observed in this study, but the top gene associated with thiopurine resistance (Table 2) was sulfite oxidase (SUOX), a mitochondrial enzyme that belongs to the same family of molybdenum oxo-transferases that includes xanthine oxidase (30). An association between SUOX and thiopurine sensitivity has not been described in the literature previously, but because the enzymatic activities of SUOX and xanthine oxidase rely on molybdenum metabolites from a common biosynthetic pathway (30), it is possible that altered SUOX expression may indirectly influence the activity of xanthine oxidase and the rate of thiopurine detoxification via modulation of molybdenum metabolite levels. Also highly relevant for resistance mechanisms to this class of drug was the positive correlation of ABC1 with thiopurines (Table 2). ABC1 is a ubiquitously expressed efflux pump known to transport the products of phase II enzyme xenobiotic detoxification (31). Because the important thiopurine-converting gene TPMT codes for a phase II detoxification enzyme, this suggests a

### Table 4. Top genes correlated to VCR resistance or GC-MTX reciprocity in T-ALL cell lines [correlation coefficients vs. IC50 (all $P < 0.005$)]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Title</th>
<th>VCR</th>
<th>Gene</th>
<th>Title</th>
<th>GC-MTX reciprocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA1G1</td>
<td>Protein kinase, AMP-activated, γ</td>
<td>0.87</td>
<td>NM225012</td>
<td>Unknown</td>
<td>–0.87</td>
</tr>
<tr>
<td>TCTN3</td>
<td>Tectonic family member 3</td>
<td>0.85</td>
<td>PLCL1</td>
<td>Phospholipase C-like 1</td>
<td>–0.78</td>
</tr>
<tr>
<td>BATF3</td>
<td>Basic leucine zipper TF, ATF-like3</td>
<td>0.85</td>
<td>FZD5</td>
<td>Frizzled homolog 5 (Drosophila)</td>
<td>0.82</td>
</tr>
<tr>
<td>ZNF107</td>
<td>Zinc finger protein 107</td>
<td>–0.82</td>
<td>ZNF492</td>
<td>Zinc finger protein 492</td>
<td>0.76</td>
</tr>
<tr>
<td>IPO9</td>
<td>Importin 9</td>
<td>–0.82</td>
<td>LOC150759</td>
<td>Hypothetical protein LOC150759</td>
<td>–0.79</td>
</tr>
<tr>
<td>GZMH</td>
<td>Granzyne H (cathepsin G-like 2)</td>
<td>0.82</td>
<td>BCL6</td>
<td>B-cell CLL/lymphoma 6</td>
<td>–0.78</td>
</tr>
<tr>
<td>WASF1</td>
<td>WAS protein family, member 1</td>
<td>–0.82</td>
<td>PLGLA/B</td>
<td>Plasminogen-like A /// B1 /// B2</td>
<td>–0.76</td>
</tr>
<tr>
<td>SIRT7</td>
<td>Siruin 7</td>
<td>0.82</td>
<td>COLEC10</td>
<td>Collecin subfamily 10 (C-type lectin)</td>
<td>–0.82</td>
</tr>
<tr>
<td>RAB40A</td>
<td>RAB40A, RAS oncogene family</td>
<td>–0.82</td>
<td>NDE1</td>
<td>NudE nuclear distribution gene E</td>
<td>–0.81</td>
</tr>
<tr>
<td>ZNF117</td>
<td>Zinc finger protein 117</td>
<td>–0.82</td>
<td>AGPAT2</td>
<td>Acylglycerol-phosphate acyltransferase</td>
<td>0.76</td>
</tr>
<tr>
<td>FES</td>
<td>Feline sarcoma oncogene</td>
<td>0.82</td>
<td>HNRNPA3</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
<td>–0.72</td>
</tr>
<tr>
<td>INTS6</td>
<td>Integrator complex subunit 6</td>
<td>0.81</td>
<td>ECH1</td>
<td>Enol CoA hydratase 1, peroxisomal</td>
<td>0.72</td>
</tr>
</tbody>
</table>

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previously unsuspected role for MRP1 in this detoxification pathway.

The other known drug transporter found to be significantly associated with resistance in this study was MFSD10 (major facilitator superfamily domain member 10, or TETRAN), which was significantly correlated to ARAC IC50 (Table 3). The activity of this transporter has previously been linked to the development of resistance to nonsteroidal antiinflammatory agents (32), but to our knowledge this is the first reported association of the gene with resistance to ARAC. The correlation

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**Figure 1.** Biology of resistance patterns associated with individual drugs or drug combinations in the T-ALL panel. A, the top probe sets correlating with resistance to glucocorticoid (GCs; \( P < 0.01 \) for both MPRED and DEX, 555 probe sets), thiopurines (TPs; \( P < 0.05 \) for both 6MP and 6TG, 725 probe sets), anthracyclines (ACs; \( P < 0.05 \) for both DOX and DNR, 715 probe sets), VCR (\( P < 0.01 \), 500 probe sets), MTX (\( P < 0.01 \), 695 probe sets), ASP (\( P < 0.01 \), 1073 probe sets), ARAC (\( P < 0.01 \), 427 probe sets), the 10-drug combination model (top 50 correlating probe sets from each drug, 380 nonredundant probe sets), and the GC-MTX reciprocity index (\( P < 0.05 \) for each of MPRED, DEX, and MTX but with opposite polarity for GCs and MTX, 959 probe sets), were processed with Ingenuity Pathway Analysis. The \( P \)-values for the top biological pathways associated with each of these drug combinations are represented by the density of cell shading (color = log(\( P \)-value)). B, demonstration of GC-MTX reciprocity. The top 25 probe sets positively (left graph) and negatively (right graph) correlating with MPRED IC50 across the cell line panel were correlated against each of the other drugs and their coefficients averaged. Scores above or below the dotted lines are statistically significant. Positively correlated probes set for MPRED (left) were significantly correlated with DEX resistance but negatively correlated with MTX resistance. The opposite pattern was observed for negatively correlated MPRED probe sets (right); MPRED, methylprednisolone; DEX, dexamethasone; ARAC, cytosine arabinoside; 6TG, 6-thioguanine; 6MP, 6-mercaptopurine; DNR, daunorubicin; DOX, doxorubicin; ASP, L-asparaginase; VCR, vincristine; MTX, methotrexate.
coefficients of other genes known to be involved in canonical pathways of drug resistance, multidrug resistance, detoxification, and/or metabolism are given in Supplementary Table S2. Of particular note are genes significantly correlated with multiple drugs and/or drug classes such as DGUOK (deoxyguanosine kinase, involved in the phosphorylation of purine deoxyribonucleosides) significantly correlated with resistance to glucocorticoids, ASP, anthracyclines, and VCR; GLUD1/2 (glutamate dehydrogenase, an important enzyme for glutamate metabolism and therefore cancer cell growth) associated with glucocorticoids and anthracyclines; and various genes involved with TNF signaling, such as TRAF3 (TNF receptor-associated factor 3), significantly correlated with glucocorticoids, ASP, anthracyclines, ARAC, and VCR resistance (Supplementary Table S2). The cytochrome P450 enzyme CYPA43 was significantly negatively regulated with resistance to both thiopurines and ASP (Supplementary Table S2), suggesting a previously undescribed role for this enzyme in the cellular detoxification of these agents.

Prognostic relevance of drug–gene profiles

To assess whether the T-ALL drug–gene correlates we had observed in vitro were relevant for mechanisms of resistance in primary cells, we developed a statistical model to describe the IC₅₀ profiles of our panel of cell lines using gene expression data, and incorporating the information from all 10 drugs (see Materials and Methods). We then applied this model to microarray data from the CCG-1961 T-ALL cohort (22) that we have previously published (7), to generate “resistance scores” for each drug and each patient specimen (n = 50). These in turn were used to model the probability of relapse for each patient. Importantly, this approach was able to identify patients with a significantly poorer outcome (Fig. 2A), indicating that the gene expression profiles correlated with in vitro drug resistance were also correlated to clinical outcome in patients. In contrast, the clinical features of age, white blood cell count, and gender have no significant univariate association with outcome in this relatively small cohort (7). To validate this finding, we applied the same model to 2 further T-ALL patient cohorts (33, 34), making use of array data previously published (23). Once again, the model was able to predict patients with significantly poorer outcome in both cohorts (Fig. 2B and C). The prediction accuracies for the 10-drug model across each of the cohorts are provided in Supplementary Table S4 and the logistic regression equations used in each case are in Supplementary Table S5.

Drug-contribution modeling

To assess whether, for any given patient, a prediction of relapse within our 10-drug model might be driven by particular drugs, we evaluated the relative contribution of each drug within the model toward the final designation of outcome. In the CCG-1961 T-ALL cohort, this analysis clearly indicated that the expression profiles associated with 2 drugs in particular, 6MP and MPRED, were key factors in driving the correct prediction of relapse or nonrelapse (Fig. 3A). Further analysis indicated that patients within this cohort that went on to relapse were associated at the time of diagnosis with higher resistance scores for 6MP and lower resistance scores for MPRED (Fig. 3B). We have previously reported that glucocorticoid resistance in T-ALL is associated with a proliferative metabolism (11), in contrast to many other chemotherapeutic drugs, which act more efficiently in rapidly dividing cells. Under this hypothesis, increased glucocorticoid sensitivity would therefore be consistent with slower growth (11), and this in turn would be associated with an increased resistance to other chemotherapeutic agents including 6MP, which may on balance contribute to poorer clinical outcome.

To assess whether subgroups of patients existed within this cohort for whom the correct prediction of relapse might be driven by different patterns of drug effects within the model, we conducted hierarchical clustering based on these drug-contribution profiles. This clearly identified 2 patient subgroups, which behaved distinctly (Fig. 3C). When the contribution scores for these subgroups were plotted separately it became...
apparent that although relapse predictions in one group were driven by expression profiles associated with 6MP resistance, the other group was most strongly influenced by profiles associated with MTX resistance (Fig. 3D). This indicates that 2 different drug–response profiles might underpin poor clinical outcome within this cohort.

To validate this approach we repeated the entire drug–contribution analysis using array data (23) from the independent POG-8704 (33) and POG-9404 (34) T-ALL cohorts. Interestingly, the correct prediction of relapse in each of these cohorts was influenced by distinct patterns of drug contribution. In POG-8704, subgroups were associated with ARAC, 6MP, and DNR-resistance signatures, whereas in POG-9404 ARAC and MTX profiles were the main contributors to relapse prediction (Fig. 3E–G, and Supplementary Fig. S1). Notably, POG-9404 was the only cohort for which 6MP gene expression correlates made no substantial contribution to outcome prediction (Fig. 3G), yet was the only study of the 3 in which 6MP was used through all phases of therapy including induction (34), suggesting that augmented use of this agent for all patients may have effectively removed the prognostic influence of this drug within the model.
To summarize the pattern of drug influence observed across all 3 cohorts, we calculated the overall proportion of patients for whom the correct prediction of outcome was strongly influenced by each drug or drug class (Fig. 3H). These data show that the expression signatures most useful for the correct prediction of relapse across all 3 cohorts were linked to resistance to the thiopurines (particularly 6MP). In contrast, in vitro–derived expression correlates for VCR were generally without prognostic influence in these patients (Fig. 3H).

Discussion

In this study we have showed that resistance profiles to different drugs measured in T-ALL cell lines in vitro are associated with distinct biological pathways and that these pathways have direct clinical relevance for patient outcome. The identification of reliable prognostic factors has been more difficult for T-ALL than for B-lineage ALL (35) and improved markers are needed for outcome prediction to improve T-ALL patient-risk stratification. This is exemplified in the CCG-1961 cohort where we have previously showed age, white blood cell count and gender to have no significant univariate association with outcome (7). Although the adequacy of cell lines to accurately reflect in vivo disease continues to be a matter of considerable debate (36), we have previously showed that our panel shows critical features of the primary disease, particularly in relation to drug-sensitivity profiles and patterns of growth (20, 37), and in the majority of cases unsupervised clustering of microarray data accurately pairs cell lines with the bone marrow specimens from which they were derived. Critically, the model developed in this in vitro setting has been validated using primary material from 3 independent T-ALL patient cohorts, clearly showing the clinical relevance of these drug–gene signatures.

This is not the first time that in vitro gene-expression profiles have been used to model drug-resistance and subsequent patient outcome in cancer (38–42). In particular, Lee and colleagues published a sophisticated algorithm to translate drug activities in the NCI-60 cell line panel to predict drug-sensitivity and clinical outcome in multiple cancer types (40). However, many of these published models have focused on individual agents and typically did not test the prognostic power of their classifiers in multiple independent patient cohorts. Recently 2 groups have generated large cancer cell line drug-biomarker databases, combining genome-wide sequencing approaches with high-throughput drug-library screening (43, 44) but neither of these have yet been used to generate predictive models of outcome in patient cohorts. The strongest studies in this area in the field of ALL, focused on ex vivo drug-sensitivity measurements of diagnostic patient specimens (17, 18). Although these successfully generated gene-expression models that could be validated in independent cohorts, they focused only on the 4 agents most commonly used in remission induction therapy. This study extends these findings by including interaction effects from the other drugs routinely used in consolidation and maintenance therapy.

Our results have highlighted particular genes and pathways associated with sensitivity or resistance to each of the 10 agents tested. The cell lines tested have been grown in the absence of drug selection and their sensitivity profile therefore reflects naturally occurring diversity within the panel. As such, few of the top-ranked genes represent canonical drug-resistance markers, echoing the findings from Holleman and colleagues (18), where 98% of the genes shown to be associated with resistance in primary specimens were not previously linked to classical resistance mechanisms. Instead, novel associations were revealed from our data, including a link between MLL expression and glucocorticoid resistance (10), and a potential role for sulfite oxidase (SUOX) and the drug-transporter ABCC1 (MRP1) in mediating sensitivity to thiopurines, relationships that we are currently exploring experimentally. It is clear however, both from our findings and from numerous studies in this area, that diverse pathway interactions are often associated with common resistance phenotypes. Finally, our findings have also highlighted an unexpected reciprocity between MTX and glucocorticoid resistance, pointing to a convergence of metabolic pathways for these agents and suggesting there would be clinical benefit from the use of MTX in situations where glucocorticoid resistance is observed, for example ALL patients at the time of relapse or those with poor early responses to 7-day prednisone.

One of the most important indications from this study is that 6MP may play a particularly important role in determining outcome in T-ALL. Mercaptopurine was one of the first chemotherapy agents used to treat pediatric ALL (29) and is a cornerstone of maintenance therapy along with another important antimetabolite, MTX (45, 46). Other studies have showed that thiopurines may be of particular benefit in T-ALL and called for more intensive use of this class of agent to treat this patient group (46, 47). Our findings are consistent with this commentary and would support consideration for the use of 6MP in earlier stages of T-ALL therapy, with carefully designed clinical trials required to directly test this hypothesis. In B-lineage ALL, the drugs given in the first 4 weeks of therapy (typically prednisolone, VCR, DNR, and ASP) seem to be critical because the detection of minimal residual disease (MRD) at the end of induction is predictive of long-term outcome (48). In contrast, MRD at a later time point (day 78) is more prognostic in T-ALL (48), indicating that it is the response to the drugs generally received in postinduction consolidation therapy (which includes 6MP) that are most important for T-ALL (2).

Although ex vivo testing of diagnostic specimens has shown T-ALL patients to be generally sensitive to 6MP (49), our data predicts a certain level of resistance may exist in some patients at the time of disease presentation. Because 6MP is myelosuppressive, it is often administered at the limits of tolerability and increasing dose intensity to counter resistance may not therefore be possible for many patients (2, 46). An alternative approach may be to develop adjuvant therapies that sensitize blasts to 6MP exposure. Examples of existing agents that are known to augment cellular sensitivity to 6MP include allopurinol, which inhibits the 6MP detoxifying enzyme xanthine oxidase (50), and MTX, which is well known to synergize with 6MP through a convergence of metabolic pathways (45). As a result of this latter fact, 6MP and MTX are modulated in parallel in most clinical trials, however a customized approach based on patient genotype for the enzyme TPMT (a deficiency...
of which dramatically increases the risk of mercaptopurine-induced toxicity) would better leverage the synergistic relationship of these 2 agents by retaining both at their limits of tolerability (2). Alternative approaches to sensitize blasts to 6MP, and therefore further improve response to thiopurine therapy, may be provided by targeting the genes and pathways linked to 6MP sensitivity in this study.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. H. Beesley, M. J. Firth, U. R. Kees
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc): A. H. Beesley, U. R. Kees
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. H. Beesley, M. J. Firth, D. Anderson, J. Ford

References

Writing, review, and/or revision of the manuscript: A. H. Beesley, M. J. Firth, D. Anderson, A. L. Samuels, U. R. Kees
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Ford

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In Vitro Drug–Gene Modeling for T-ALL Outcome


Drug–Gene Modeling in Pediatric T-Cell Acute Lymphoblastic Leukemia Highlights Importance of 6-Mercaptopurine for Outcome

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