In Vivo Response to Methotrexate Forecasts Outcome of Acute Lymphoblastic Leukemia and Has a Distinct Gene Expression Profile

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Abstract

Childhood acute lymphoblastic leukemia (ALL) is the most common cancer in children, and can now be cured in approximately 80% of patients. Nevertheless, drug resistance is the major cause of treatment failure in children with ALL. The drug methotrexate (MTX), which is widely used to treat many human cancers, is used in essentially all treatment protocols worldwide for newly diagnosed ALL. Although MTX has been extensively studied for many years, relatively little is known about mechanisms of de novo resistance in primary cancer cells, including leukemia cells. This lack of knowledge is due in part to the fact that existing in vitro methods are not sufficiently reliable to permit assessment of MTX resistance in primary ALL cells. Therefore, we measured the in vivo antileukemic effects of MTX and identified genes whose expression differed significantly in patients with a good versus poor response to MTX.

Methods and Findings

We utilized measures of decreased circulating leukemia cells of 293 newly diagnosed children after initial “up-front” in vivo MTX treatment (1 g/m²) to elucidate interpatient differences in the antileukemic effects of MTX. To identify genomic determinants of these effects, we performed a genome-wide assessment of gene expression in primary ALL cells from 161 of these newly diagnosed children (1–18 y). We identified 48 genes and two cDNA clones whose expression was significantly related to the reduction of circulating leukemia cells after initial in vivo treatment with MTX. This finding was validated in an independent cohort of children with ALL. Furthermore, this measure of initial MTX in vivo response and the associated gene expression pattern were predictive of long-term disease-free survival (p < 0.001, p = 0.02).

Conclusions

Together, these data provide new insights into the genomic basis of MTX resistance and interpatient differences in MTX response, pointing to new strategies to overcome MTX resistance in childhood ALL.

Trial registrations: Total XV, Therapy for Newly Diagnosed Patients With Acute Lymphoblastic Leukemia, http://www.ClinicalTrials.gov (NCT00137711); Total X, IIIB, Phase III Randomized Study of Antimetabolite-Based Induction plus High-Dose MTX Consolidation for Newly Diagnosed Pediatric Acute Lymphocytic Leukemia at Intermediate or High Risk of Treatment Failure (NCI-T93-0101D); Total XIIIBL, Phase III Randomized Study of Antimetabolite-Based Induction plus High-Dose MTX Consolidation for Newly Diagnosed Pediatric Acute Lymphocytic Leukemia at Lower Risk of Treatment Failure (NCI-T93-0103D).

The Editors’ Summary of this article follows the references.
Introduction

Childhood acute lymphoblastic leukemia (ALL), the most common cancer in children, can now be cured in approximately 80% of patients [1,2]. Pharmacogenomics aims to elucidate the genomic determinants of treatment response and why treatment fails to cure the remaining 20% of patients, many of whom have favorable prognostic features [3,4]. Prior studies have provided insight into the genomic determinants of resistance to several antileukemic agents [5], but methodological constraints have precluded such genome-wide studies of in vitro methotrexate (MTX) resistance. This research gap is unfortunate because MTX is widely used in the treatment of many human cancers, including essentially all treatment protocols for newly diagnosed ALL [1].

The pharmacokinetics and pharmacodynamics of MTX in ALL cells are well understood, whereas the genomic determinants of the antileukemic effects of MTX remain to be elucidated [6,7]. Cellular uptake of MTX is mediated by the protein reduced folate carrier [8], whereas its efflux is mediated by ATP-binding cassette (ABC), subfamily C 1 (ABCC1) and ABCC4 [9–11]. MTX is a tight-binding inhibitor of its primary target, enzyme dihydrofolate reductase (DHFR), which disrupts cellular folate metabolism [12]. Within leukemic cells, MTX is metabolized into poly(γ-glutamate) forms (MTXPGs) by an adenosine triphosphate (ATP)-dependent reaction catalyzed by folylpolyglutamate synthetase [13]. Compared to their monoglutamate form, MTXPGs are retained longer in cells because they are not readily effluxed by ABC transporters [14,15]. MTXPGs are more potent inhibitors of other target enzymes such as thymidylate synthetase, glycinamide ribonucleotide transformylase, and aminomimidazole carboxamidetransformylase. These enzymes are involved in biosynthetic pathways that are critical for DNA synthesis, DNA repair, and cell replication [14,15]. Furthermore, accumulation of MTXPG has also been shown to differ among major ALL subtypes [16] and to influence treatment response and outcome in childhood ALL [17–19].

A more complete understanding of the mechanisms of MTX resistance in ALL cells is needed if new treatment strategies are to be developed for patients whose leukemia is resistant to this important component of ALL chemotherapy [6]. Prior genomic studies of ALL chemotherapy resistance have not focused on MTX [5,20,21], because the resistance of primary ALL to MTX cannot be accurately measured by in vitro methods such as the MTT assay [22]. For this reason, we used the in vivo response of newly diagnosed patients to initial single-agent MTX treatment, measured as an initial decrease in circulating ALL cells, to quantitate the antileukemic effects of MTX. We then aimed to identify genes whose expression in primary ALL cells is significantly related to the in vivo antileukemic effects of MTX.

Methods

Patients and Genetic Characterization of Leukemia Cells

A total of 293 children aged 18 y or younger with newly diagnosed ALL, enrolled on the St. Jude Total Therapy XIII and XV protocols, were included in this study (Figures 1 and S1). The investigation was approved by the Institutional Review Board at St. Jude Children’s Research Hospital, and signed informed consent was obtained from parents or legal guardians before enrollment. Patient characteristics (race, sex, age, pretreatment white blood cell count [WBCPRE], ALL subtype) were assigned by investigators at St. Jude Children’s Research Hospital. Race, sex, and age were determined by questionnaire; WBCPRE, ALL subtype were determined according to the clinical protocol. The diagnosis of ALL was based on morphology, cytochemical staining, and immunophenotyping of blast cells for classification as B cell lineage or T cell lineage, as previously described [23–28]. The only patients excluded were those who did not have a diagnosis of ALL, or were aged < 1 y or > 18 y, or were given ALL treatment prior to referral to SJCRH.

After stratification for age, WBCPRE, immunophenotype, and sex, patients were randomized to receive initial intravenous treatments of high-dose MTX (HDMTX; 1 g/m²) either as HDMTX4H (HDMTX by infusion over 4 h; n = 108), as HDMTX24H (HDMTX by infusion over 24 h; n = 125), or as HDMTX24H+MP (HDMTX by infusion over 24 h plus mercaptopurine [MP] 1 g/m² by intravenous injection; n = 60). All patients who received allopurinol less than 72 h prior to HDMTX were excluded from the analyses because of potential effects on de novo purine synthesis. Circulating leukemia cells were measured before therapy (WBCPRE) and at day 3 following start of HDMTX treatment (WBCDay3), prior to the administration of other antileukemic agents. Leukocyte counts were determined with a Coulter counter (model F_STKR; Coulter, Hialeah, Florida, United States).

Isolation of ALL Blasts from Bone Marrow Aspirate

ALL blasts were obtained from bone marrow aspirates at diagnosis and 42–44 h following treatment. Samples consisted of 5–10 ml of bone marrow collected in syringes containing 800 units of heparin and kept on ice until processed. Leukemic cells were obtained by density separation over a Ficoll-Hypaque gradient and washed three times with a solution of HEPES, Hanks buffered solution, and heparin, as previously described in detail [18].

RNA Extraction and Gene Expression Profiling of Diagnostic Bone Marrow ALL Cells

Of the 293 patients treated with up-front HDMTX, 161 had sufficient diagnostic ALL cells for gene expression analysis (i.e., had sufficient leukemia cells in their diagnostic bone marrow aspirates to permit RNA isolation from 5 × 10⁶ to 1 × 10⁷ ALL cells). High-quality total RNA was extracted with TriReagent (MRC, Cincinnati, Ohio, United States) from cryopreserved mononuclear cell suspensions from bone marrows at diagnosis. Total RNA was processed and hybridized to the HG-U133A oligonucleotide microarray (Affymetrix, Santa Clara, California, United States). This array contains 22,215 gene probe sets, representing approximately 12,357 human genes, plus approximately 3,820 expressed sequence tag clones with unknown function [29]. Following removal of probe sets with >95% absent calls, 13,488 probe sets remained. Scaled expression values of all probe sets were log2 transformed to stabilize variance.

Additional information on the microarray methods and results can be found at http://www.stjude.org/data/.
Determination of MTX Polyglutamylated Metabolites in Bone Marrow ALL Cells 42–44 Hours Post-treatment

Intracellular MTXPGs were extracted from 42- to 44-h post-treatment bone marrow ALL cells kept in a buffered solution (Tris, EDTA, and 2-mercaptoethanol [pH 7.8]) by first boiling (100 °C for 10 min), then stored frozen at −80 °C until analysis. The HPLC separation and the radioenzymatic quantitation of MTX and six polyglutamylated metabolites (MTXPG2–7) were performed as previously described [30,31]. These MTXPG measurements were available for 230 patients. All results were expressed as picomoles of MTX or MTXPG per 10⁹ cells.

Statistical Analysis and Bioinformatics

MTX responses as measured by WBC\textsubscript{Day3}, WBC\textsubscript{PRE}, and MTXPG values were logarithmically transformed to normalize their respective distributions. The Pearson correlation test was applied in order to determine the association between WBC\textsubscript{Day3} and ALL subtype, MTXPG, and WBC\textsubscript{PRE}. The difference between WBC\textsubscript{PRE} and WBC\textsubscript{Day3}, WBC\textsubscript{ADay3} (the WBC residual based on the linear regression of log[WBC\textsubscript{PRE}] change to log[WBC\textsubscript{Day3}]) was determined by taking the residuals of the linear regression model of WBC\textsubscript{Day3} versus WBC\textsubscript{PRE}, which was available for 293 patients. Specifically, MTX response is defined as:

\[
\text{WBC Day3} = 0.492 \times \log(\text{WBC PRE}) - \log(\text{WBC Day3}) + 0.0229
\]

We indicated “MTX poor response” and “MTX good response” in Figures 2 and 3 according to the cutoff for good responders (WBC\textsubscript{Day3} < 0.14) and poor responders (WBC\textsubscript{Day3} > 0.14), based on the bottom and top quartile of 293 patients.

Data were available for 161 patients on both WBC\textsubscript{Day3} and gene expression in diagnostic bone marrow leukemia cells (Figure S1). The association between each individual probe
set and WBC\textsubscript{D3} was determined using the Pearson correlation test. Gene probe sets were rank-ordered by their \( p \)-values. Final gene probe sets were selected based on a false discovery rate (FDR) cutoff of 1.5%.

For each patient, we computed a gene expression profile using the weighted average of the expression signals of top selected genes. The Pearson correlation coefficient between each gene’s expression and WBC\textsubscript{D3} was used as the weight. This weighted average of expression signals was used as the summary of the top gene expression profile for each patient. Specifically, the gene expression profile was computed according to the following formula:

\[
\text{Gene Expression Score} = \sum_{j=1}^{50} \text{weight}_j \times \text{gene expression}_j
\]

Where \( j = 1 \ldots 50 \) top selected gene probe sets as listed in Table S3, and weights were determined as the correlation coefficients as listed in the same table.

We compared the top 50 gene profile with the top 100 gene profile; the correlation coefficient was 0.989 (\( p < 0.001 \), Pearson correlation test). These tests were performed using standard statistical functions in R software.

We tested 37 GenMAPP pathways and 319 Gene Ontology–biological process (GO–BP) gene groupings for association with WBC\textsubscript{D3} using the “globaltest” method [32] implemented in the R Bioconductor package [33]. This test was used to infer over-representation of specific biological pathways, and the “geneplot” function was applied to plot the importance of selected genes with default parameters. Multiple testing was adjusted using the Bonferroni method and the FDR according to Storey and Tibshirani [34].

Cell Cycle Distribution

The percentage of ALL cells in S-phase was determined in diagnostic bone marrow aspirates from patients for whom an adequate number of cells were available (\( n = 154 \)). Propidium iodide–stained DNA content was measured by flow cytometry using the Coulter EPICS V flow cytometer (Coulter Electronics, Hialeah, Florida, United States), and the computer program ModFit (Verity Software House, Verity, Topsham, Maine, United States) was used to calculate the percentages of cells in G0/G1, S, and G2/M phase.

Treatment Outcome Analysis

The duration of disease-free survival (DFS) was defined as the time from diagnosis until the date of leukemia relapse (event), or the last follow-up (censored). Second malignancies and death due to other reasons were censored at the time of...
occurrence. Treatment outcome was available in 136 patients of the 293 patients treated with HDMTX with WBCPRE and WBCday3 measured. Of note, patients treated on protocol T15 were excluded because of short follow-up. Time was also censored at the last follow-up date if no failure was observed. Single-variable analysis using Cox proportional hazards regression, as modified by Fine and Gray [35] was used to estimate the relative risk of an event. Significant associations from the single variable analyses were further evaluated in a multiple variable analysis, which included risk classification, age, lineage, and ALL subtype in addition to WBCday3 and the top 50 gene expression profile. DFS curves were calculated by reversing the cumulative incidence curve, where MTX poor responders represent the top quartile, intermediate responders the middle two quartiles, and good responders the bottom quartile.

Results

Relation among WBCday3, WBCPRE, Treatment, ALL Subtype, and MTX Metabolism

Patient characteristics (race, sex, age, WBCPRE, ALL subtype) were similar among patients randomly assigned to receive HDMTX4H, HDMTX24H, or HDMTX24H+MP (p > 0.13, Figure S2). Furthermore, there was no difference in WBCday3 among the three randomized treatment groups (Table 1). The lack of differences among treatment groups coupled with our previous findings of minimal de novo purine synthesis (DNPS) inhibition and antileukemic effects of a single dose of intravenous MP [36], allowed us to analyze patients treated with HDMTX4H, HDMTX24H, and HDMTX24H+MP as a single group, to enhance the statistical power of our analyses.

WBCday3 was significantly lower than WBCPRE (n = 293, p < 0.0001) and was highly correlated with WBCPRE (p < 0.0001, r = 0.79, Figure 2). Therefore, to remove the effect of the pretreatment leukemia burden (WBCPRE) we used the WBCday3 corresponding to the residuals from the linear regression model of WBCday3 versus WBCPRE (Figure 2). As indicated in Figure S3, the histogram of the residuals approximates a normal distribution, in contrast to the skewed distribution of percentage drop in WBC count from diagnosis to day 3 (WBCday3drop; p = 0.1 versus p < 0.001, Kolmogorov-Smirnov test). There was a statistically significant association between WBCday3 and polyglutamylated MTX levels (MTXPG2–7) in ALL cells (n = 230, p = 0.0001, r = −0.25, Figure 4), with higher MTXPG2–7 associated with greater antileukemic effect. There was not a significant relation between WBCday3 and ALL subtype (n = 293, p = 0.07).

Relation among WBCday3, Gene Expression, and Pathway Analysis

Our analyses of antileukemic effects after in vivo MTX treatment and gene expression in pretreatment ALL cells identified the 50 most significant gene probe sets that were associated with antileukemic effect of MTX (WBCday3; Figure 3). The FDR was less than 1.5% for these gene probe sets, and each gene had a Pearson correlation coefficient higher than 0.3 or lower than −0.3 and a p-value less than 0.001. Among these genes, the expression patterns for 21 were positively and 29 were negatively related to MTX response. Genes significantly associated with MTX response included those involved in nucleotide metabolism (thymidylate synthetase [TYMS] and CTP synthase [CTPS]), cell proliferation and apoptosis (B-cell CLL/lymphoma 3 [BCL3], centromere protein F [CENPF], cell division cycle 20 [CDC20], abnormal spindle-like [ASPM], transforming, acidic coiled-coil containing protein 1 [TACC1]) and DNA replication (cell division cycle 60B1 [CDC60B1], cell division cycle 60B2 [CDC60B2], centromere protein A [CENPA], centromere protein H [CENPH]).

Table 1. Patient Characteristics Were Not Different among the HDMTX Treatment Groups

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<th>Category</th>
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<th>p-Valuea</th>
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aChi-square test.
bReference group.
doi:10.1371/journal.pmed.0050083.t001
significant correlation of WBC\textsubscript{Day3} with the total MTXPG level in ALL cells from 230 patients (i.e., a higher total MTXPG concentration is associated with a better in vivo MTX response) \((p = 0.0001, r = -0.25, \text{Pearson correlation})\).

\textbf{Figure 4.} Scatterplot of WBC\textsubscript{Day3} Versus Level of Total MTXPGs

There is a significant correlation of WBC\textsubscript{Day3} with the total MTXPG level in ALL cells from 230 patients (i.e., a higher total MTXPG concentration is associated with a better in vivo MTX response) \((p = 0.0001, r = -0.25, \text{Pearson correlation})\).

[Image 45x28 to 63x45]

\textit{Antileukemic MTX Genomics and Outcome}

Relation between Disease-Free Survival and Expression of \textit{CTPS, TYMS, DHFR, MTX Response (WBC\textsubscript{Day3})}, and Top 50 Gene Expression Profile

The median follow-up of patients for this analysis was 9.1 years from diagnosis, comprising patients enrolled in St. Jude Total Therapy XIII protocol \((n = 136)\). WBC\textsubscript{Day3} and \textit{TYMS} and \textit{DHFR} expression, were related to DFS according to Cox proportional hazards regression analyses that compared patients who remained in continuous complete remission with those who relapsed during the follow-up period. The univariable analysis of variables potentially related to DFS revealed significance for expression of \textit{TYMS} (hazard ratio \([HR] = 0.6; p = 0.008\)), expression of \textit{DHFR} \((HR = 0.41; p = 0.015)\); MTX response \((WBC\textsubscript{Day3}, HR = 21.5; p < 0.001)\), and the top 50 gene expression profile \((HR = 1.09; p = 0.02; \text{Table 3})\), but not for the expression of \textit{CTPS} or the WBC\textsubscript{Day3}.

Patients with the best MTX response (i.e., bottom quartile of the residual WBC\textsubscript{Day3}) had significantly better 5-y DFS compared to patients with the worst response (top quartile) \((\text{DFS} \pm SE = 96.9\% \pm 3.1\% \text{ versus } 81.4\% \pm 7\%; p = 0.033 \text{ for quartiles compared}; \text{Figure 5A})\). Likewise, patients with a gene expression profile indicative of a good MTX response (bottom quartile of the gene expression profile) had significantly better 5-y DFS compared to patients with a gene expression profile indicative of poor MTX response (top quartile of the gene expression profile) \((5-y \text{ DFS} \pm \text{variance: } 87.5\% \pm 6.9\% \text{ versus } 72\% \pm 9.2\%; p = 0.019 \text{ for quartiles compared}; \text{Figure 5B})\). Patients with higher \textit{TYMS} or \textit{DHFR} expression (i.e., top quartile of the expression level) had significantly better 5-y DFS compared to patients with lower \textit{TYMS} or \textit{DHFR} expression (i.e., bottom quartile of the expression level) \((5-y \text{ DFS} \pm \text{variance: } 87.9\% \pm 5.8\% \text{ versus } 72.7\% \pm 7.9\%; p = 0.024 \text{ for } \text{TYMS} \text{ and } 93.5\% \pm 4.5\% \text{ versus } 69\% \pm 8.1\%; p = 0.041 \text{ for } \text{DHFR} \text{ for quartiles compared}; \text{Figure 5C})\).

Furthermore, multivariable Cox regression analysis (Tables 3 and 2) that also included the conventional National Cancer Institute ALL risk criteria (i.e., ALL subtype, age, and WBC at diagnosis) revealed significance for MTX response \((WBC\textsubscript{Day3}, HR = 22.6; p = 0.0046)\), and the expression of \textit{TYMS} \((HR = 0.58; p = 0.044)\) and \textit{DHFR} \((HR = 0.51; p = 0.019)\). The top 50 gene expression profile did not reach statistical significance in predicting relapse in the overall population when other known risk factors were included, although the trend remained evident \((p = 0.08, \text{Tables 3 and 2})\).

Discrimination of MTX Response Using the Top 50 Gene Expression Profile and Assessment in an Independent Validation Cohort

In an independent test set of 18 additional patients who received initial HDMTX according to the St. Jude Total Therapy XV protocol, we performed gene expression analysis at diagnosis and determined WBC (ALL cell) count at diagnosis and on day 3. The gene expression profile of the top 50 genes was significantly related to the residual WBC\textsubscript{Day3} in this patient cohort (top 50 gene profile, \(p = 0.0065, r = 0.62, \text{Pearson correlation}; \text{Figure 6A})\), thus
observed WBC Day3 values are significantly closer to the WBC Day3 squared was 1.042 using the WBC predictor values using WBC Day3 after initial MTX treatment based on the known WBC PRE in these 18 newly enrolled patients. For that, we used either the WBC ADay3 linear regression function or the median WBC%drop developed in the original test cohort of 293 patients. The sum of daily differences between the observed and the predicted values using WBC ADay3 squared was 1.042 using the WBC Day3 squared was 1.042 using the WBC Day3 linear regression model and 3.35 using the median WBC%drop. The observed WBC Day3 values are significantly closer to the predicted values using WBC ADay3 (Figure 6B) than those based on WBC%drop (Figure 6C; \( p = 0.0025 \), paired \( t \)-test), thereby further indicating that WBC ADay3 is a more accurate measure of in vivo MTX response than WBC%drop.

### Discussion

The current studies have identified genes that are expressed at a significantly different level in acute lymphoblastic leukemia cells of patients who exhibit a poor in vivo response to HDMTX. High-throughput genomic approaches to assess the expression levels of RNA transcripts in cancer cells are providing new insights into pathogenesis, classification, diagnosis, stratification, and prognosis of many human cancers [23,37–39]. The drug resistance and gene expression profiles of leukemia cells have also been used to identify genes related to the sensitivity of ALL cells to several antileukemic agents and to forecast differences in treatment response [5,20,21]. These findings have also revealed novel targets for the discovery of new agents to reverse drug resistance, such as our prior discovery of MCL1 over-expression in glucocorticoid-resistant ALL [5], and the subsequent discovery that rapamycin can down-regulate MCL1 expression and increase sensitivity of leukemia cells to dexamethasone [37,40]. However, prior to the current study, there has not been a comprehensive analysis of genes related to the antileukemic effects of MTX in primary leukemia cells.

We therefore evaluated MTX response in vivo after initial therapy, because this is the only possible time to assess the antileukemic effects of MTX as a single agent in patients and because there are no reliable in vitro methods. Thus, our study focused on treatment-naive ALL, and assessed de novo resistance. This revealed that WBC ADay3 was a superior measure of in vivo MTX response when compared to the percentage drop in leukemia cells (i.e., WBC%drop), and that WBC ADay3 was predictive of long-term DFS. Furthermore, the difference in survival cannot simply be explained by differences in MTX systemic exposure (Figure S6).

To better understand the biological basis underlying MTX response in ALL patients, we used an unbiased genome-wide approach to identify genes whose expression in primary leukemia cells in vivo was significantly related to WBC ADay3. This process revealed 48 genes and two cDNA clones that are highly related to the in vivo MTX response (WBC ADay3); even after adjusting for MTX PG accumulation (\( n = 230 \)) (Table S3). Among those genes significantly associated with MTX response were genes involved in nucleotide metabolism (TYMS and CTPS), cell proliferation and apoptosis (BCL3, CDC20, CENPF, and FAIM3), and DNA replication or repair (POLD3, RPA3, RNASEH2A, RPMI, and H2AFX). The antileukemic effects of MTX involve inhibition of purine and pyrimidine synthesis, and the current findings indicate that interindividual differences in nucleotide synthesis influence the in vivo antileukemic effects of MTX. This finding was confirmed by a global test analysis that identified the nucleotide biosynthesis pathway as one of the most discriminating biological pathways related to MTX response. Significance of the global test was largely explained by three key genes (TYMS, DHFR, and CTPS) belonging to the nucleotide biosynthesis pathway.

Our analysis also showed that low expression of DHFR, TYMS, and CTPS was significantly correlated with poor in vivo MTX response [6,41,42]. It has been shown that DHFR, TYMS, and CTPS expression is associated with critical biological processes such as DNA synthesis and cell proliferation [43,44], a finding consistent with low expression of these genes reflecting a decrease in the number of ALL cells in S-phase. As MTX selectively affects cells in the S-phase of the

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<th>Parameter</th>
<th>( r )</th>
<th>( p )-Value</th>
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<tr>
<td>WBC ADay3</td>
<td>-0.20</td>
<td>0.013</td>
</tr>
<tr>
<td>Top 50 gene profile</td>
<td>-0.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBC ADrop</td>
<td>0.034</td>
<td>0.67</td>
</tr>
</tbody>
</table>

### Table 2. Pearson Correlation of Selected Biological and Response Parameters with Percentage of Cells in S-Phase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Univariable Hazard Analysis</th>
<th>Multivariable Cox Proportional Hazard Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>( p )-Value</td>
</tr>
<tr>
<td>WBC ADay3</td>
<td>21.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TYMS</td>
<td>0.60</td>
<td>0.008</td>
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<tr>
<td>DHFR</td>
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<td>0.015</td>
</tr>
<tr>
<td>Top 50 gene profile</td>
<td>1.09</td>
<td>0.02</td>
</tr>
</tbody>
</table>

### Table 3. Univariable Hazard Analysis of the Risk of Relapse with Variables Related to Initial In Vivo MTX Response and Multivariable Cox Proportional Hazard Analyses Each Including Known Prognostic Factors (i.e., ALL Subtype, Age at Diagnosis, Risk Group)
The possibility that genetically determined high expression of the known folate metabolism genes in pretreatment ALL cells is less important in causing de novo MTX resistance than previously thought. It may well be that these folate pathway genes are important for the acquired drug resistance that emerges during MTX treatment. It is also plausible that expression or function of these proteins is not reflected by the level of their mRNA expression in ALL cells. These possibilities merit further investigation, which is beyond the scope of the current work.

Defining the genomic determinants of ALL resistance to individual antileukemic agents is essential if the pharmacogenomics of drug resistance are to be elucidated, because the current and prior studies have shown that genes discriminating drug resistance in ALL are drug specific [5,48]. To assess whether the genes we identified as related to de novo MTX resistance reflect a global resistance phenotype versus a MTX-specific effect, we compared the previously reported gene expression profiles for ALL resistance to PVAD (prednisone, vincristine, asparaginase, and daunorubicin), with the top 50 genes discriminating MTX response in the current study. This comparison revealed no overlap in the genes related to MTX resistance and the 124 genes related to prednisone, vincristine, asparaginase, or daunorubicin PVAD resistance [5]. This result indicates that genes identified in the current study are not a marker of general drug resistance or a global predictor of survival, rather they are specific to MTX (or perhaps other antifolates, but not all ALL chemotherapy). Furthermore, we applied our MTX gene expression profile to the publicly available German/Dutch dataset [5], and documented that the MTX gene expression profile is not related to prednisolone response (Figure S5, Table 2). This finding does not preclude the possibility that genetically determined high expression of the known folate metabolism genes in pretreatment ALL cells is less important in causing de novo MTX resistance than previously thought. It may well be that these folate pathway genes are important for the acquired drug resistance that emerges during MTX treatment. It is also plausible that expression or function of these proteins is not reflected by the level of their mRNA expression in ALL cells. These possibilities merit further investigation, which is beyond the scope of the current work.

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sensitivity in this independent patient cohort (unpublished data).

Among the 50 genes that were expressed at a significantly different level in leukemia cells of MTX good responders versus poor responders, 29 were overexpressed in the MTX poor- responders. It is plausible that these overexpressed genes would be candidate targets for small molecules or other strategies to down-regulate their function, as a means to modify MTX response. Such a strategy has already proven successful in finding agents to modify the sensitivity of ALL cells to steroids [40], and it is plausible that specific inhibitors of genes overexpressed in leukemia cells resistant to MTX could be viable targets for modulating the antileukemic effects of MTX. Likewise, strategies to invoke the expression of genes that are underexpressed in MTX poor responders could be tested for their ability to modulate MTX sensitivity.

The current study is the first, to our knowledge, to identify genes whose expression is related to in vivo MTX response in patients with newly diagnosed ALL. Our data provide new insights into the genomic basis of interpatient differences in MTX response and point to new strategies for overcoming de novo MTX resistance in childhood ALL. In addition, our data indicate that early treatment response to MTX is a significant prognostic indicator for long term DFS in children with ALL.

Supporting Information

Figure S1. Flow Diagram of Data and Methods Used
Gray boxes indicate data used for the analyses, white boxes intermediate data, shaded boxes data analysis method used, and n the number of patients.

Figure S2. Box Plot of WBCDay3 Versus MTX Treatment Group
The three initial treatment groups with HDMTX were not different in their WBCDay3 (HDMTX24H, n = 125; HDMTX24H+MP, n = 60; HDMTX4H, n = 108, Welch two-sample t-test).

Figure S3. Histogram of WBC Change and WBC Residuals
Shown are the distributions of 293 patients for (A) WBCLogChange that is defined as log(WBCpre) minus log(WBCday3), p = 0.04, i.e., is significantly different from a normal distribution; and (B) WBCDay3 that is the residuals of the (log(WBCpre) minus log(WBCday3)) linear regression, p = 0.10, i.e., is not significantly different from a normal distribution.

Figure S4. Gene Plot of the Gene Ontology Nucleotide Biosynthesis Pathway
The gene plot gives a bar and a reference line for each gene probe set categorized for this pathway. The bar indicates the influence of each probe set on the correlation with MTX response (WBCday3). If the height of the bar exceeds the reference line the probe set is significantly related to MTX response. Marks indicate the standard deviations by which the bar exceeds the reference line. Red indicates gene probe sets with a positive correlation and green indicates gene probe sets with a negative correlation with MTX response.

Figure S5. Scatterplot of TYMS and DHFR Expression, WBCDay3, and Top 50 Gene Profile Versus Percentage of Leukemia Cells in S-Phase
Shown are the scatterplots for 154 patients correlating percentage of leukemia cells in S-phase with (A) the expression of TYMS and DHFR (respectively, r = 0.59; p < 0.001; r = 0.39, p < 0.001, Pearson correlation); and (B) for WBCDay3 and the residual WBCday3 of the WBC change (respectively, r = 0.034, p = 0.67; r = 0.20, p = 0.013, Pearson correlation); and (C) the top 50 gene expression profile (r = −0.57, p < 0.001, Pearson correlation).

Figure S6. MTX Systemic Exposure Was Not Different among Responder Groups
There was no difference (p = 0.82) in MTXauc (methotrexate area under the curve, representing MTX systemic exposure) in the groups (MTX good responder [GR] versus MTX intermediate [IR] versus MTX poor responder [PR]). We used the same cutoff for MTX response (WBCDay3) as in Figure 3. Therefore, the difference in survival cannot be explained by the difference in MTXauc.

Table S1. The Top Ten GenMAPP (of 37) (A) and GO–BP (of 319) (B) Pathways Associated with WBCDay3
The column titled “probe-set correlations with WBCDay3” indicates whether most probe sets in the pathway have a positive correlation, a negative correlation or a mixture of positive and negative correlations with WBCDay3.

Table S2. Multivariable Cox Proportional Hazard Analysis of the Risk of Relapse
Individual factors related to MTX response are highlighted in gray (i.e., WBCDay3, TYMS, DHFR, top 50 gene profile), each including known prognostic factors (i.e., ALL subtype, age at diagnosis, risk group).

Table S3. The Top 50 Probe Sets Associated with WBCDay3

Text S1. CONSORT Checklist
Found at doi:10.1371/journal.pmed.0050083.sd001 (64 KB DOC).
Accession Numbers
MIAME-compliant primary microarray data are available through the Gene Expression Omnibus (NCBI) at http://www.ncbi.nlm.nih.gov/geo/ under GSE10255 and GSM258912–GSM259072.

Acknowledgments
We thank the patients and their parents for their participation in this study; our clinical staff for facilitating protocol-based patient care; and our research nurses, Sheri Ring, Lisa Walters, Terri Kiecher, Margaret Edwards, Vickey Simmons, and Paula Condy. We also thank Yaqin Chu, May Chung, Emily Melton, and Siama Saheyl for outstanding technical assistance; and Nancy Kornegay and Mark Wilkinson for computer and database expertise.

Author contributions. MJS, C-HP, MHC, and WEE designed the experiments and the study. MJS, DP, WY, GC, C-HP, MVR, MHC, and WEE contributed to writing the paper. MHC collected gene expression and patient data for this study.

Abbreviations. ABC, ATP-binding cassette; ALL, childhood acute lymphoblastic leukemia; DFS, disease-free survival; FDR, false discovery rate; GO–BP, Gene Ontology–biological process; HDMTX, high-dose methotrexate; HR, hazard ratio; MTX, methotrexate; MTXPG, methotrexate polyglutamate; WBC% drop, percent WBC count change; WBC, white blood cell count; WBClog(WBCPRE) change to log(WBC Day3); WBCDay3, peripheral WBC defined by the WBC residual based on the linear regression of

References
After methotrexate treatment (a result confirmed in an independent study), patients before treatment. They found that the expression of 50 genes was associated with methotrexate of nearly 300 patients with newly diagnosed ALL. In this study, the researchers measured the expression of these genes in leukemic cells shortly after the first methotrexate dose and a specific gene expression profile indicative of a good response were more likely to be alive after 5 years than patients with the worst methotrexate response and the poor-response gene expression profile.

What Do These Findings Mean? These findings provide important new insights into the genetic basis of methotrexate resistance in newly diagnosed childhood ALL and begin to explain why some patients fail to respond to this drug. They also show that the reduction in circulating leukemic cells shortly after the first methotrexate dose and a specific gene expression profile both predict the long-term survival of patients. These findings also suggest new ways to modulate sensitivity to methotrexate. Down-regulation of the expression of the genes that are underexpressed in methotrexate poor responders and so improve the chance of responding to methotrexate. Alternatively, it might be possible to find ways to increase the expression of the genes that are expressed more highly in poor responders than in good responders to improve patient responses to methotrexate. Additionally, it might be possible to find ways to increase the expression of the genes that are underexpressed in methotrexate poor responders and so improve the chance of responding to methotrexate.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0050083.

- The US National Cancer Institute provides a fact sheet for patients and caregivers about ALL in children and information about its treatment (in English and Spanish)
- The US Leukemia and Lymphoma Society also provides information for patients and caregivers about ALL
- The Children’s Cancer and Leukaemia Group (a UK charity) provides information for children with cancer and their families
- MedlinePlus provides additional information about methotrexate (in English and Spanish)