Prognostic Role of the Reduced Folate Carrier, the Major Membrane Transporter for Methotrexate, in Childhood Acute Lymphoblastic Leukemia: A Report from the Children’s Oncology Group

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Abstract

Purpose: The value of measuring expression of individual genes relevant to particular chemotherapy drugs and encoding metabolizing enzymes, transporters, or drug targets, as predictors of treatment response and outcome in pediatric acute lymphoblastic leukemia (ALL), remains controversial.

Experimental Design: In a case-control population of 91 pediatric B-precursor ALL patients (42 relapsed within 4 years [cases] and 49 did not relapse [controls]), we used real-time reverse transcription-PCR to measure transcript levels for 20 genes relevant to chemotherapy with the five major drugs used to treat this disease, including asparaginase, 6-mercaptopurine, methotrexate, prednisone, and vincristine. Results were confirmed in a separate case-control population of 26 patients.

Results: Only the human reduced folate carrier (hRFC) gene, encoding the major membrane transporter for methotrexate, showed a significant difference in median transcript levels between the 42 cases and the 49 controls (P = 0.0278, Wilcoxon test). Using cutoffs for hRFC expression levels (based on Akaike information criterion), there were statistically significant associations between hRFC transcripts and treatment relapse (P = 0.0052). hRFC-B, corresponding to the major hRFC transcript form in ALL, was also measured by real-time reverse transcription-PCR and was prognostic. The association between treatment relapse and hRFC levels was validated in a separate study population of 14 cases and 12 controls from an earlier case-control study (P = 0.0221).

Conclusions: Our results strongly suggest the prognostic importance of hRFC gene expression to treatment outcomes in pediatric ALL. They validate our previous studies of hRFC transcriptional regulation in pediatric ALL and provide further compelling evidence for the critical role for methotrexate in the successful treatment of this disease.

The development of curative therapy for children diagnosed with acute lymphoblastic leukemia (ALL) is a paradigm for the evolution of a successful therapy of cancer. Ever since Farber et al. (1) first showed >50 years ago that the antifolate aminopterin could induce clinical remissions in pediatric ALL, there have been increasing numbers of survivors so that today ~80% of children are cured (2, 3). It is remarkable that much of this improvement is not so much due to new drugs for treating ALL as to a better understanding of how to use combinations of existing drugs (e.g., asparaginase, 6-mercaptopurine, methotrexate, prednisone, and vincristine) in intensified regimens and in treating presymptomatic central nervous system–disseminated ALL.

An underlying principle of modern ALL therapy involves the use of risk-adapted therapy, in which the intensity of therapy administered is based on the risk of relapse (2). Accordingly, patients considered at higher risk based on age and white blood cell (WBC) count at diagnosis, gender, and immunophenotype, as well as the presence or absence of prognostically important genotype or cytogenetic features, are treated with more intensive chemotherapy. Conversely, low-risk patients are typically treated with less intensive therapies. Patient subgroups with T-cell ALL or ALL with rearranged MLL and BCR-ABL are viewed as having unfavorable outcomes, and patients with B-precursor (BP)-ALL with t(12;21) or hyperdiploidy are generally considered to have a good prognosis (3).

However, in spite of the success of risk-based therapy for ALL, a significant number of patients continue to relapse and eventually die of this disease. Thus, identification of additional factors that contribute to treatment failure is important.
molecular markers predictive of treatment response and outcome are sorely needed. With this reasoning, several recent studies attempted to identify prognostically relevant genes in ALL by correlating in vitro drug sensitivities or treatment outcomes to chemotherapy drugs with gene expression profiles on oligonucleotide microarrays (4–8). Although several genes were identified, which were subsequently confirmed independently in patient populations (6, 7), there was little overlap with gene expression “signatures” for established subgroups of patients, such as t(12;21), hyperdiploid BP-ALL, or T-cell ALL, or with established drug resistance genes.

An alternative approach involves emphasis on individual genes associated with “pathways” unique to particular chemotherapy drugs and encoding metabolizing enzymes, transporters, or drug targets. Previous studies examined an association between expression of several genes relevant to the major drugs used in ALL therapy and drug resistance, or risk of relapse or outcome following chemotherapy (9–19). However, in general, results have been mixed and no clear picture has yet emerged of individual pathway genes that are especially prognostic and/or that can be used to predict treatment responses a priori.

On this basis, we designed a case-control study of 91 pediatric BP-ALL patients, approximately half of whom failed therapy within 4 years of diagnosis. Our initial goal was to use real-time reverse transcription-PCR methods to rigorously assess patterns of gene expression for 20 genes encoding proteins involved with transport, metabolism, therapeutic targets, or apoptosis signaling and considered to be closely associated with success or failure of chemotherapy with the five major drugs used to treat ALL (i.e., asparaginase, 6-mercaptopurine, methotrexate, prednisone, and vincristine). As described herein, our results strongly suggest a uniquely prognostic role for the human reduced folate carrier (hRFC), the major transport protein for methotrexate in pediatric ALL (20). Results for hRFC were confirmed in a separate case-control BP-ALL population from an earlier study (21). This surprising result further documents the critical role that methotrexate plays in ALL therapy and strongly validates our previous studies of hRFC transcriptional and posttranscriptional regulation in this disease (22, 23).

Materials and Methods

**Patient specimens.** Two groups of patient specimens were obtained from the Children’s Oncology Group ALL cell bank and used for this study. (a) One hundred diagnostic specimens (including approximately equal numbers of cases and controls) were obtained from BP-ALL patients treated on the Pediatric Oncology Group (POG) protocols, including POG 8602 (13 cases and 13 controls), POG 8698 (1 case and 1 control), POG 9005 (1 case and 6 controls), POG 9006 (10 cases and 7 controls), POG 9201 (2 cases and 3 controls), POG 9406 (12 cases and 15 controls), and POG 9605 (3 cases and 4 controls). Ninety-one of these samples were usable, including 42 cases and 49 controls. (b) Twenty-six diagnostic specimens were from our earlier study (21), including controls (n = 12) and cases (n = 14) from patients treated on the POG 8602 protocol, in which total RNAs were available. Patient cohort b had no overlap with patients from POG 8602 in cohort a. For both study groups, controls who remained in remission for 4 or more years following diagnosis, and cases who were children who suffered bone marrow relapses within 4 years of diagnosis. For study group a, cases were matched to controls by risk groups; for study group b, cases were matched to controls by risk groups and treatment arms. With all chemotherapy protocols, the main drugs used were i-asparaginase, 6-mercaptopurine, methotrexate, prednisone, and vincristine. Patients received i.v. methotrexate (1-2.5 g/m2) over 24 h as part of consolidation therapy, and methotrexate (20 mg/m2) was given i.m. during maintenance therapy. Patients who died in remission within 4 years of diagnosis were excluded from the study.

Leukemia blasts were purified by standard Ficoll-Hypaque density centrifugation. To identify t(12;21) from our cohort of BP-ALL patient samples, TEL-AML1 transcripts were reverse transcribed and cDNAs were PCR amplified on a LightCycler Real-time PCR machine (Roche, Indianapolis, IN) with forward (5′-AACCCCTCACCCTCTCCTAC-3′) and reverse (5′-TGAGACCGGCGGTTGAAGC-3′) primers. Sample handling and data analysis protocols were approved by the Committee on Investigation Involving Human Subjects at Wayne State University and by the Children’s Oncology Group ALL Biology Subcommittee.

**Real-time reverse transcription-PCR analysis of gene expression profiles.** Analyses of gene expression were done in a blinded manner. Total RNAs were extracted from primary ALL lymphoblasts using the RNEasy Midiprep kit (Qiagen, Valencia, CA). cDNAs were prepared from 1 μg RNAs using random hexamers and a reverse transcription-PCR kit (Applied Biosystems, Foster City, CA) and purified with the QiAquick PCR Purification kit (Qiagen). Transcript levels for 20 genes, for the hRFC-A1/A2 and hRFC-B noncoding exons, and 18S rRNA levels were quantitated using a LightCycler Real-time PCR machine. Reactions contained 2 μL of purified cDNA or standard plasmid, 4 mmol/L MgCl2, 0.5 μmol/L each of sense and antisense primers, and 2 μL FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche) as described previously (22, 23). Specificity of the real-time PCR amplifications was confirmed by melting curve analysis and comparisons to melting curves for standard templates. Primers and PCR conditions are summarized in Table 1. For each gene of interest, external standard curves were constructed using serial dilutions of linearized templates, prepared by amplification from suitable CDNA templates, subcloning into a TA-cloning vector (i.e., PCRII-TOPO vector, Invitrogen, Carlsbad, CA), and restriction digestion.

**Statistical methods.** All data analyses were done using the SAS System (24) and R (25). The data for transcript levels ranged over an order of magnitude for each of the 20 genes, and some were highly skewed. Therefore, statistical analyses reported were done on log-transformed gene activity levels. The nonparametric Wilcoxon test and Kruskal-Wallis test were used for comparisons of transcript levels between various subgroups (cases versus controls, National Cancer Institute risk group, age group, WBC, hyperdiploidy, TEL/AML1 status, and race). For certain analyses, transcript levels were also categorized into low and high levels by choosing an appropriate cutoff level by inspection of plots of the Akaike Information Criterion (AIC; data not shown) for logit models of the probability of failure. For each possible cutoff value, a logistic model was fit, in which the probability of failure depended on the level of gene activity classified as either high or low, according to whether gene activity was higher or lower than the cutoff. AIC was plotted versus the cutoff value, with AIC values closer to zero indicating a better cutoff choice. Inspection of the receiver operating characteristic curves (data not shown) provided further insight into the reasonableness of the cutoff value chosen and whether the gene had predictive value. The associations between similarly determined high or low transcript levels of a gene and other prognostic factors (age group, WBC group, National Cancer Institute risk group, hyperdiploidy status, gender, and TEL/AML1 status) were tested using Fisher’s exact test.

**Results**

**Analysis of gene expression profiles in a case-control population of 91 BP-ALL patients.** The case-control study population included 91 children with BP-ALL, 42 of whom relapsed within 4 years of diagnosis (cases) and 49 of whom remained in...
remission for 4 or more years following diagnosis (controls). Patient age ranged from 1.1 to 18.8 y (median, 4.68 y) and WBC counts ranged from 1.3 × 10^9 to 980 × 10^9 cells/L (median, 51.0 × 10^9 cells/L). Other patient characteristics, including gender, genotype, or karyotype characteristics [t(12;21), hyperdiploidy, t(1;19), and t(2;22)] are summarized in Table 2.

The initial focus of our investigation was to measure transcript levels for 20 genes relevant to chemotherapy with the major drugs that were used for all patients (asparaginase, 6-mercaptopurine, methotrexate, prednisone, and vincristine) and encoding transporters, drug-metabolizing enzymes, drug targets, or apoptosis signaling proteins. Genes of interest included the following: (a) dihydrofolate reductase, (b) hRFC, (c) folylpolyglutamate synthetase, (d) γ-glutamyl hydrolase, (e) thiopurine s-methyltransferase, (f) hypoxanthine guanine phosphoribosyltransferase, (g) asparagine synthetase, (h) glucocorticoid receptor, (i) MDR1 (ABC1), (j) ABC1 (MRP1), (k) ABC2 (MRP2), (l) ABC3 (MRP3), (m) ABC4 (MRP4), (n) ABC5 (MRP5), (o) ABCG2 (breast cancer resistance protein), (p) B-cell leukemia/lymphoma 2, (q) B-cell leukemia/lymphoma X long isoform, (r) microtubule-associated protein 4, (s) β-tubulin class 1, and (t) β-tubulin class 3. Total RNAs were isolated from the 91 lymphoblast specimens, reverse transcribed, and amplified...
by real-time PCR using gene-specific primers (Table 1). For all genes, a broad range of transcript levels was detected, from slightly >1,100-fold for ABCB2 to ~100-fold for β-tubulin class 1 (Table 3).

Given the strength of our case-control study design, we looked into the relationship between transcript levels for the 20 genes and relapse within 4 years of diagnosis. Interestingly, only hRFC showed transcript levels that were significantly different (median, 2.0-fold; \( P = 0.0278 \), Wilcoxon test; Fig. 1) with a lower median level in the 42 cases compared with that of the 49 controls.

hRFC transcript levels were categorized as low or high based on a cutoff determined from an AIC plot. Using this cutoff, the 49 controls showed somewhat increased hRFC levels (median, 5.76 RU; range, 0.31-102.79 RU). However, this difference was not statistically significant (\( P = 0.0278 \), Wilcoxon test). As expected, BP-ALL patients identified as hyperdiploid (\( n = 21 \)) showed somewhat increased hRFC levels (median, 5.76 RU; range, 0.54-120.48 RU) over nonhyperdiploid BP-ALL patients (\( n = 70 \); median, 4.99 RU; range, 0.31-102.79 RU). However, this difference was not statistically significant.

### Treatment Outcome

Treatment outcome (relapsed versus not) and hRFC transcript levels (low versus high). Thus, 36 of 42 (85.7%) cases had low hRFC transcripts, whereas only 28 of the 49 (57.1%) controls showed low hRFC transcripts (\( P = 0.0052 \); Fig. 2, top).

Because >90% of total hRFC transcripts in pediatric ALL involves just two of the six hRFC promoters and upstream noncoding regions (designated A1/A2 and B; ref. 22), we measured relative levels of hRFC-A1/A2 and hRFC-B transcripts in this ALL cohort using primers to the hRFC-A1/A2 and hRFC-B 5’ noncoding regions. Median levels for hRFC-A1/A2 and hRFC-B transcripts were 3.03 and 8.89 relative units (RU) for the 91 BP-ALL specimens, a distribution consistent with our previous findings of relative hRFC noncoding exon usage (22, 23). hRFC-B transcript levels were again categorized into low and high groups using a cutoff from an AIC plot. With this cutoff, 39 of 42 (92.9%) cases showed low levels of hRFC-B transcripts, whereas 28 of 49 (57.1%) controls showed low levels of hRFC-B transcripts (\( P = 0.0001 \); Fig. 2, bottom). Although a similar trend was seen for the hRFC-A1/A2 transcripts (i.e., 34 of 42 cases showed low hRFC-A1/A2 levels and 32 of 49 controls showed low hRFC-A1/A2), this difference was not statistically significant (\( P = 0.1063 \); data not shown).

No statistically significant associations were found between hRFC transcript levels and age group, WBC level, race, or National Cancer Institute risk group. Although median hRFC transcripts did not seem to depend on gender (\( P = 0.1486 \), Wilcoxon test), higher transcript levels for females were suggested by the Kolmogorov-Smirnov test (\( P = 0.0452 \)). As expected, BP-ALL patients identified as hyperdiploid (\( n = 21 \)) showed somewhat increased hRFC levels (median, 5.76 RU; range, 0.54-120.48 RU) over nonhyperdiploid BP-ALL patients (\( n = 70 \); median, 4.99 RU; range, 0.31-102.79 RU). However, this difference was not statistically significant.

### Table 2. Patient characteristics for BP-ALL cohort

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Cases (( n = 42 ))</th>
<th>Controls (( n = 49 ))</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29</td>
<td>24</td>
<td>53</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>25</td>
<td>38</td>
</tr>
<tr>
<td>t(12;21)</td>
<td>4</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>40</td>
<td>78</td>
</tr>
<tr>
<td>t(9;22)</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>41</td>
<td>49</td>
<td>90</td>
</tr>
<tr>
<td>t(1;19)</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>47</td>
<td>85</td>
</tr>
<tr>
<td>Hyperdiploid</td>
<td>Yes</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>37</td>
<td>33</td>
</tr>
</tbody>
</table>

### Table 3. Gene expression data for 91 BP-ALL patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcripts (RU)*</th>
<th>Controls (( n = 49 ))</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>0.15 (0.01-1.70)</td>
<td>0.27 (0.01-5.64)</td>
<td>0.364</td>
</tr>
<tr>
<td>MDR1</td>
<td>24.81 (1.87-487.24)</td>
<td>35.67 (0.79-281.84)</td>
<td>0.360</td>
</tr>
<tr>
<td>ABCC1</td>
<td>15.10 (0.47-57.59)</td>
<td>16.01 (0.35-56.96)</td>
<td>0.846</td>
</tr>
<tr>
<td>ABCC2</td>
<td>2.25 (0.04-15.63)</td>
<td>0.93 (0.02-22.21)</td>
<td>0.145</td>
</tr>
<tr>
<td>ABCC3</td>
<td>0.66 (0.08-7.49)</td>
<td>0.70 (0.04-4.80)</td>
<td>0.550</td>
</tr>
<tr>
<td>ABCC4</td>
<td>3.98 (0.27-30.01)</td>
<td>5.26 (0.19-26.76)</td>
<td>0.632</td>
</tr>
<tr>
<td>ABCC5</td>
<td>8.53 (0.53-73.84)</td>
<td>8.74 (0.22-39.81)</td>
<td>0.978</td>
</tr>
<tr>
<td>hRFC (total)</td>
<td>3.40 (0.45-21.09)</td>
<td>6.85 (0.31-120.48)</td>
<td>0.028</td>
</tr>
<tr>
<td>DHFR</td>
<td>6.91 (0.47-52.06)</td>
<td>9.32 (0.39-79.22)</td>
<td>0.508</td>
</tr>
<tr>
<td>FPGS</td>
<td>13.32 (0.72-40.92)</td>
<td>15.99 (0.36-57.86)</td>
<td>0.426</td>
</tr>
<tr>
<td>GGH</td>
<td>0.35 (0.02-9.38)</td>
<td>0.48 (0.02-8.62)</td>
<td>0.368</td>
</tr>
<tr>
<td>ASNS</td>
<td>1.50 (0.08-12.89)</td>
<td>2.15 (0.06-9.39)</td>
<td>0.660</td>
</tr>
<tr>
<td>BCL2</td>
<td>11.54 (0.94-84.32)</td>
<td>18.54 (0.42-85.92)</td>
<td>0.248</td>
</tr>
<tr>
<td>BCL-5L</td>
<td>6.12 (0.21-40.75)</td>
<td>7.76 (0.15-34.72)</td>
<td>0.615</td>
</tr>
<tr>
<td>GCR</td>
<td>37.60 (2.22-175.21)</td>
<td>44.46 (0.65-187.79)</td>
<td>0.473</td>
</tr>
<tr>
<td>HPRT</td>
<td>3.23 (0.36-15.42)</td>
<td>4.82 (0.10-22.52)</td>
<td>0.293</td>
</tr>
<tr>
<td>MAP4</td>
<td>4.46 (0.15-30.10)</td>
<td>5.24 (0.09-23.43)</td>
<td>0.972</td>
</tr>
<tr>
<td>TPMT</td>
<td>2.93 (0.23-33.53)</td>
<td>4.45 (0.09-27.86)</td>
<td>0.304</td>
</tr>
<tr>
<td>TUBB1</td>
<td>69.34 (6.13-272.39)</td>
<td>87.93 (3.02-323.02)</td>
<td>0.615</td>
</tr>
<tr>
<td>TUBB3</td>
<td>0.05 (0.01-3.10)</td>
<td>0.07 (0.01-1.11)</td>
<td>0.632</td>
</tr>
</tbody>
</table>

**Note:** \( n \) is the number of patients studied. Relative transcript levels for the target genes were measured by real-time reverse transcription-PCR as described in Materials and Methods. Gene abbreviations are summarized in the legend in Table 1. The nonparametric Wilcoxon test was used for comparisons of transcript levels between cases and controls and the \( P \) values are reported in the table. *Value expressed as median (range).
poorer long-term prognosis than for BP-ALL, accumulates substantially lower levels of methotrexate polyglutamates (28, 29). To elucidate molecular mechanisms that potentially account for differences in methotrexate polyglutamate accumulations between ALL subtypes, Kager et al. (17) recently used oligonucleotide microarrays to analyze folate pathway gene expression profiles in diagnostic leukemia cells. Although specific alterations in expression of hRFC, ABCC1, ABCG2, and folypolyglutamate synthetase genes were identified between ALL subtypes with characteristic differences in prognosis, no attempt was made to correlate patterns of gene expression with treatment outcomes.

ALL is an ideal disease to study clinical drug resistance. This reflects the existence of well-defined end points of treatment response and the comparative ease of obtaining pure lymphoblast populations for laboratory studies. With this in mind, we began a systematic study of expression patterns for genes relevant to chemotherapy response to the five major agents commonly used in therapy of children with BP-ALL. We focused on 20 genes, including genes encoding targets, transporters, metabolizing enzymes, and apoptosis proteins.

Remarkably, for our 91-patient cohort of BP-ALLs, only hRFC among 20 drug resistance/response genes tested showed 

\[ P = 0.2725 \text{ by Wilcoxon test} \]. Although there was a suggested association between hRFC levels and t(12;21) status for BP-ALL patients identified as TEL-AML1 positive [median values of 8.74 versus 4.86 RU for TEL-AML1–positive patients \((n = 13)\) and TEL-AML1–negative patients \((n = 78)\), respectively], this was not statistically significant \((P = 0.1061, \text{ Wilcoxon test})\). There were too few patients with t(1;19) \((n = 6)\) or t(9;22) \((n = 1)\) translocations to explore associations with hRFC levels.

**Validation of hRFC findings in a separate study population.** As validation of our finding that hRFC transcript levels directly correlated with chemotherapy outcome in BP-ALL patients treated with methotrexate, we studied RNAs isolated from a separate cohort of BP-ALL patients treated with POG 8602 from our earlier study (21), for which cases \((n = 14)\) and controls \((n = 12)\) were matched by risk groups and treatment arms. In this study group, total hRFC transcripts were again significantly increased in the controls compared with the cases (increase of 3.84-fold, by median values; \(P = 0.0221\); Fig. 3), thus validating our major findings from the 91-patient cohort.

**Discussion**

In spite of extensive studies into mechanisms of drug response and resistance in tissue culture models, only rarely have these mechanisms been unequivocally confirmed in clinical disease. Probably, the best example involves the antifolate methotrexate, an agent long used for chemotherapy of pediatric ALL (2, 26). Thus, accumulations of high levels of methotrexate polyglutamates required for sustained drug activity are associated with a good prognosis in BP-ALL (27). T-cell ALL, an ALL subtype with an increased risk of relapse and accumulation of low levels of methotrexate polyglutamates, has a poorer prognosis than BP-ALL and other ALL subtypes (28, 29). To elucidate molecular mechanisms that potentially account for differences in methotrexate polyglutamate accumulations between ALL subtypes, Kager et al. (17) recently used oligonucleotide microarrays to analyze folate pathway gene expression profiles in diagnostic leukemia cells. Although specific alterations in expression of hRFC, ABCC1, ABCG2, and folypolyglutamate synthetase genes were identified between ALL subtypes with characteristic differences in prognosis, no attempt was made to correlate patterns of gene expression with treatment outcomes.

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transcript levels that were significantly different for patients who relapsed within 4 years of diagnosis (cases) and those who did not (controls). When hRFC transcripts were categorized into low versus high levels, a statistically significant association was found between treatment failure and low hRFC transcripts. Because this was a case-control study with an approximately equal number of failures and nonfailures (i.e., overall event-free-survival at 4 years is ~50%), formal comparisons of event-free-survival between patients with low and high expression levels were not done because this would not reflect the true outcomes in the pediatric BP-ALL population, in general, where the overall outcome is ~80% at 6 years (2, 3). Our results, nonetheless, strongly suggest a prognostically important role for hRFC in children with BP-ALL. Importantly, hRFC levels in our study population seemed to be completely independent of patient age and WBC.

As with any correlative study, it is important to validate the major findings in a separate group of patients. When we assayed hRFC transcripts in a separate case-control cohort of 26 BP-ALL patients treated on a single clinical protocol from our earlier study (21), essentially identical results were obtained to those from the larger group of patients treated on multiple protocols. This establishes the generality of our findings in pediatric BP-ALL and also shows that these associations between hRFC expression and treatment outcome are not treatment protocol-dependent.

A prognostic role for hRFC in pediatric BP-ALL would seem especially appropriate given its central role in the cellular uptake of methotrexate. Methotrexate remains an important component of modern treatment protocols for pediatric BP-ALL, in connection with both intensification and maintenance therapies (2, 3, 26). In addition, methotrexate is administered intrathecally in the prophylaxis and treatment of CNS leukemia. Impaired transport via hRFC frequently occurs accompanying exposure of human and rodent cells to antifolate drugs in vitro (30, 31) and in murine tumor cells in vivo after chemotherapy with methotrexate (32). Further, alterations in levels of hRFC were associated with clinical methotrexate resistance or decreased event-free-survival in both adult and pediatric leukemias (13, 20) and in osteosarcoma patients (33). In BP-ALL lymphoblasts, hRFC transcripts are generally proportional to methotrexate uptake capacity (34) and, as further confirmed in this report, hRFC transcripts in de novo ALLs span an extraordinarily broad range (22, 23, 34). The latter may, in part, reflect differences in hRFC gene expression among subgroups of ALL (e.g., BP-nonhyperdiploid, BP-hyperdiploid, TEL-AML1, and E2A-PBX1; ref. 17). However, we were unable to confirm these associations in our patient cohort, most likely due to the case-control design of our study and/or insufficient patient numbers.

Recent studies on hRFC gene structure and regulation have begun to shed light on the molecular bases for variations reported in hRFC expression in primary ALL specimens (22, 23, 34). Transcription of hRFC in both BP- and T-cell ALL involves but two (A1/A2 and B) of the six major noncoding hRFC exons and promoters (22, 23). Although Sp1, USF1, and GATA1 levels are critical determinants of hRFC transcription in ALL, neither DNA methylation nor promoter polymorphisms contribute to differences in hRFC expression (23). In the present study, hRFC-B transcripts (corresponding to ~70% of the total) in BP-ALL patients showed nearly identical associations with treatment relapse as did total hRFC. Although a similar trend was detectable for hRFC-A1/A2 transcripts, this association was not statistically significant. This may reflect greater variability in hRFC-A1/A2 expression between patients, inefficient translation and high rates of transcript turnover of hRFC-A1/A2 transcripts compared with hRFC-B transcripts (35) or, possibly, the synthesis of the NH₂-terminally modified hRFC-A1/A2 protein isoform initiated from an upstream AUG in the A1/A2 noncoding sequence (at position −192; ref. 22). The hRFC-A1/A2 protein is characterized by slightly altered transport properties from wild-type hRFC (translated from the major translation start site at position +1). Thus, both transcriptional and posttranscriptional controls are likely to be important determinants of steady-state hRFC levels and function in ALL. The net effect of these mechanisms is to generate sufficient hRFC protein and methotrexate transport in ALL lymphoblasts for high levels of dihydrofolate reductase inhibition and methotrexate polyglutamate synthesis following administration of methotrexate (36). In ALL lymphoblasts, even small changes in methotrexate transport by hRFC would have profound effects on pharmacologic activity due to the extremely low levels of dihydrofolate reductase and hRFC proteins compared with levels in most cultured cells (34).

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References


Clinical Cancer Research

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