Polymorphism G<sub>80</sub>A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia

Caroline Laverdière, Sonia Chiasson, Irina Costea, Albert Moghrabi, and Maja Krajnović

Methotrexate (MTX) is a key component of chemotherapeutic regimens used in the treatment of childhood acute lymphoblastic leukemia (ALL). Resistance to this drug may arise by, among other factors, altered cellular uptake that may hamper the efficacy of the treatment. Recently, a G<sub>80</sub>A polymorphism has been described in the reduced folate carrier gene (RFC1), which encodes the major MTX transporter. Here, we assessed the association between the genetic polymorphisms G<sub>80</sub>A and both MTX plasma levels and childhood ALL outcome. Children with the A<sub>80</sub> variant had worse prognoses than patients with the GG genotype (P = .04), as shown by event-free survival estimates. Patients homozygous for A<sub>80</sub> had higher levels of MTX (P = .004) than the other genotype groups. Possible explanations for observed associations are discussed; however, additional experiments are required to achieve understanding of the underlying mechanism. (Blood. 2002;100: 3832-3834)

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Brief report

Polymorphism G<sub>80</sub>A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia

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Methotrexate (MTX) is a key component of chemotherapeutic regimens used in the treatment of childhood acute lymphoblastic leukemia (ALL), the most frequent malignancy in the pediatric population. The reduced folate carrier gene (RFC1) is a major MTX transporter whose impaired function was recognized as a frequent mechanism of antifolate resistance. Different genetic alterations affecting RFC1 transport properties were found in cell lines selected for antifolate resistance and in patient lymphoblasts. Recently, a G<sub>80</sub>A polymorphism, which replaces His by Arg at position 27 of the RFC1 protein, was identified. The G variant correlated with lower plasma folate and higher homocysteine levels in healthy persons and was found at higher frequency in children with neural tube defects. Because folate and homocysteine homeostasis are affected by MTX action, it is possible that RFC1 G<sub>80</sub>A may also modulate the outcome in patients treated with this drug. In the present study, we analyzed the association between the RFC1 G<sub>80</sub>A polymorphism and both disease outcome and MTX plasma level in children with ALL.

Study design

Patients

The patients (n = 204) included in this study were children of French Canadian origin who had ALL and were treated at the Sainte-Justine Hospital in Montreal, QC, Canada between 1988 and 2000. Multigent chemotherapy protocols used were DFCI 87-01, DFCI 91-01, and DFCI 95-01, all developed by the Dana-Farber Cancer Institute. Patient samples were obtained at diagnosis after informed consent was provided according to the Declaration of Helsinki. Approval for this study was obtained from the institutional review board of the Hôpital Sainte-Justine. Patient characteristics and clinical prognostic factors at diagnosis (sex, age, white blood cell count [WBC], ALL cell type, treatment protocol, risk group, and DNA index) are given in Table 1. Each patient enrolled in this retrospective study received a high MTX dose (4 g/m<sup>2</sup>) during the induction phase. MTX was also given intrathecal for central nervous system treatment (6-12 mg, depending on age) and at a once-a-week dose of 30 mg/m<sup>2</sup> during the maintenance phase. Children who had relapses or fatal outcomes from the disease were defined as having an event. To reduce MTX toxicity, leucovorin rescue therapy (200 mg/m<sup>2</sup> followed by 24 mg/m<sup>2</sup> every 6 hours until routinely measured plasma MTX levels were 0.1 μM or lower) was given after high-dose MTX. MTX plasma levels were thus available at 3 time points (24, 36, and 48 hours) for the 73 children for whom RFC1 genotyping was also performed.

Genotyping and MTX plasma levels

RFC1 amplification of patient DNA isolated from buccal epithelial cells, peripheral blood, or bone marrow in remission was obtained as described elsewhere. Polymerase chain reaction–restriction fragment-length polymorphisms (PCR-RFLP; HaeII digestion) was used to optimize allele-specific oligonucleotide (ASO) hybridization assay (Figure 1A) subsequently used for the screening of patient DNA. Standard conditions were applied, except that ASOs specific for the G<sub>80</sub> (5’ gcacacgaggTgccg) or the A<sub>80</sub> (5’ gcacacgaggTgcgg) RFC1 variant were designed and hybridized at 45°C with PCR products immobilized on a membrane. Measurement of MTX plasma levels was performed by fluorescence polarization immunoassay (TDx Abbott Laboratories, Chicago, IL) according to the manufacturer’s instructions.

Statistics

Differences in the frequencies of genotypes dichotomized as carriers of RFC1 A<sub>80</sub> variant versus noncarriers between children with and without an event, and those with or without the prognostic factors listed in Table 1, were assessed by χ<sup>2</sup> analysis. For event-free survival (EFS) analysis, survival time corresponded to the time between diagnosis and the event (n = 35). For patients without an event (censored cases, n = 169), it corresponded to the end of follow-up (5 years after diagnosis).
treatment) or to February 2002 (patients who received treatment or who entered the follow-up period). Overall time to the event and of follow-up ranged from 1 to 84 months (interquartile range, 26-84 months); the median was 49.5 months. Differences between EFS obtained by Kaplan-Meier for the patients with and without the RFC1 A80 variant were determined using a log-rank test. The impact of the RFC1 A80 variant on the EFS probabilities was estimated by Cox regression analysis with the enclosure of prognostic factors that influenced ALL outcomes in this group of patients and by applying forced entry of all variables or stepwise routine. The influence of the patients’ characteristics on EFS was assessed by Kaplan-Meier and Cox regression analyses.

Repeated measures analysis of variance (ANOVA) was used to compare MTX levels (μM) based on the 3 time-points between children with and without event and among patients with different RFC1 genotype categories. For that purpose, log-transformed values of MTX level were used because of its skewed distribution. All analyses were performed by SPSS version 10.00.

## Results and discussion

A significant difference in the frequency of RFC1 genotypes was observed between children with and without an event (Table 2). Carriers of the RFC1 A80 variant had a higher risk for events than were those with the GG genotype (odds ratio [OR] = 3.0; 95% CI,
Table 2. Distribution of RFC1 G80A polymorphism among ALL patients with and without event

<table>
<thead>
<tr>
<th>RFC1</th>
<th>Event no. (%)</th>
<th>Non-event no. (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>5 (14.3)</td>
<td>56 (33.1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>20 (57.1)</td>
<td>78 (46.2)</td>
<td>2.9 (1.0-8.1)</td>
<td>.05</td>
</tr>
<tr>
<td>AA</td>
<td>10 (28.6)</td>
<td>35 (20.7)</td>
<td>3.2 (1.0-10.1)</td>
<td>.05</td>
</tr>
</tbody>
</table>

OR indicates odds ratio; CI, confidence interval.
For carriers of the AA variant, OR = 3.0; 95% CI, 1.1-8.1; P = .03.

1.8-8.1; P = .03). Similarly, Kaplan-Meier analysis showed that carriers of the A80 variant had the worst ALL outcomes (P = .04; Figure 1B). In Cox regression analysis, hazard ratio (HR) estimates for patients with RFC1 A80 retained their significance (HR = 2.8; 95% CI, 1.0-8.1; P = .05) in the presence of other prognostic factors, which also influenced ALL outcome (age, WBC, type of protocol, and risk classes; Table 1). When the initial Cox regression model was applied further to stepwise analysis, RFC1 genotype and age appeared to have the highest predictive value for an event (P = .05 and P = .03 respectively). We did not find any correlation between RFC1 genotypes and patient characteristics listed in Table 1.

We next analyzed the influence of RFC1 polymorphism on MTX plasma levels (Figure 1C) and found a significant association (P = .02), which was mainly caused by higher MTX plasma levels in the patients with AA than in those with other genotypes (P = .004). However, we did not observe the association between MTX levels and disease outcome (P = .6, data not shown).

Although modest, the association between RFC1 polymorphism and ALL outcome suggests that this variant might contribute to the estimation of ALL prognosis. The finding of this study is in agreement with the reports of others who suggest the functional impact of this polymorphism: G80A influenced folate/homocysteine levels, and correlated with neural tube defects, especially among children whose mothers reported low folate intake. The amino acid change (strong to weak basic amino acid) in a first transmembrane domain (TMD1) caused by G80A substitution was expected to alter RFC1 transport properties. Several alterations in TMD1 in cell lines selected for MTX resistance were shown to change the ratio of RFC1 affinities of MTX versus other folate substrates. Likewise, the carriers of A might have lower MTX affinity (and, presumably, higher MTX levels, as shown in this study) and higher affinity for other folate substrates (and higher level of folate cofactors, as shown in other studies).

On the other hand, MTX level, which shows high pharmacokinetic variability, may be influenced by different factors, such as hepatic or renal function, and is not a reliable indicator of the transport function. In addition recent in vitro studies in erythroleukemia cell lines showed no difference in MTX transport between the G and the A RFC1 variant, whereas only a minor (2-fold) difference in transport of 5' formyl tetrahydrofolate cofactor was found. Therefore, additional studies are needed to explain the underlying mechanism linking RFC1 polymorphism and ALL outcome. A prospective study assessing intracellular MTX levels and RFC1 substrate binding affinities in patients with and without RFC1 A80 variant is under way in our laboratory. It would also be important to assess the relative impact of RFC1 polymorphism on ALL outcome with regard to other variants relevant for MTX response.

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References