Folate cycle gene variants and chemotherapy toxicity in pediatric patients with acute lymphoblastic leukemia

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Methotrexate (MTX) is an important component of consolidation and maintenance therapy of childhood acute lymphoblastic leukemia (ALL). Nevertheless, a certain number of patients can develop resistance or adverse drug effects, which may hamper the efficacy of treatment or require drug dose reduction and drug withdrawal. Following the hypothesis that gene variants of the MTX action pathway can affect outcome of ALL, we analyzed a number of polymorphisms in the genes of the folate cycle for their impact on reduced event-free survival (EFS). Thymidylate synthase (TS) is inhibited by glutamylated forms of MTX. A repeat polymorphism is described in the enhancer region of this gene with a triple repeat (3R) increasing gene transcription and TS levels. A G80A polymorphism of the reduced folate carrier (RFC1) gene, encoding a major MTX transporter, results in an amino-acid replacement. A G80A polymorphism of the reduced folate carrier (RFC1) gene, encoding a major MTX transporter, results in an amino-acid replacement in the RFC1 domain, which is important in folate/antifolate binding. The A870G polymorphism in the cyclin D1 (CCND1) gene modulates mRNA splicing and altered CCND1 expression was previously shown to play a role in the development of MTX resistance. The substrate for methylene tetrahydrofolate reductase (MTHFR), 5,10-methylene-tetrahydrofolate, and its derive, 10-formyl-tetrahydrofolate, whose formation is dependent on the methylene tetrahydrofolate dehydrogenase (MTHFD1), are essential cofactors for de novo thymidylate and purine synthesis. Two polymorphisms in the MTHFR gene, a C→T and an A→G substitution, result in amino-acid replacements causing reduced enzymatic activity, whereas a G1958A substitution in the MTHFD1 gene leads to an amino-acid change within the 10-formyl-tetrahydrofolate synthetase domain. Recently we described reduced EFS for childhood ALL patients who are homozygous for the TS 3R or CCND1 A870 variant as well as for carriers of at least one MTHFR T677 variant or RFC1 A80 allele. The role of TS 3R and CCND1 A80 homozygosity, as well as that of MTHFR T677 allele in poorer childhood ALL outcome was recently confirmed by others. Here we hypothesized that variants associated with reduced EFS can also correlate with a lower incidence of toxicity in patients with childhood ALL.

Design and Methods

The ALL patients were treated at Sainte-Justine Hospital with one of three treatment protocols of the Dana-Farber Cancer Institute (87-01, 91-01 and 95-01). Data were available for 186 patients out of 200 consecutive patients, all of European descent (age range 1-18 years), who were previously assessed for the relationship between genotypes and EFS. These patients and their disease characteristics, as well as details of treatment have been previously described. In brief, MTX was given once a week at a dose of 12 mg/m².
dose of 30 mg/m² during consolidation and maintenance in all three protocols. Dose modification guidelines were also the same across the three protocols.

Estimates of toxicity to bone marrow and liver function were based on the reduction of white blood cells (leukopenia), platelets (thrombocytopenia) and absolute neutrophil count, and elevation of liver enzymes (alanine aminotransferase and aspartate aminotransferase). Laboratory tests were performed weekly, 7 days after MTX administration. The mean number of weeks assessed per patient was 80. Toxicity was graded using the common criteria for adverse events of the National Cancer Institute. Grades 3 and 4 were considered for all parameters except for thrombocytopenia for which grade 2 was also included. Thrombocytopenia grade 3 and 4 or liver toxicity grade 4 occurred rarely, and in such cases grades 3 and 4 were combined whereas the remaining parameters were considered separately for the analysis (Table 1). Previously obtained genotypes were used for the analysis; the details of the genotyping have been described elsewhere. Information on MTX dose during maintenance treatment was available in the same patients and the correlation between drug doses and genotypes was reported previously.

This study was approved by the Ethics Committee of Sainte-Justine Hospital and the research was conducted in accordance with the Declaration of Helsinki.

**Statistics**

Genotypes are analyzed as dichotomous variables in accordance with their influence on EFS. The genotype grouping includes either carriers of minor alleles (MTHFR 677, MTHFR 1298, MTHFD1 A516G, and RFC1 A20 variants) or homozygous individuals for TS SR and CCND1 A1290 allele who were compared to patients without these genotypes. The frequencies of weeks with high-grade hematologic and liver toxicity was compared using the Mann-Whitney test. The proportion of weeks with a particular toxicity between all patients with and without given genotypes was compared using the χ² test, and accompanied by the genotype-associated rate ratio.

**Results and Discussion**

The frequencies of weeks with high-grade hematologic and liver toxicity that developed following MTX administration during consolidation and maintenance treatment are outlined in Table 1. The inter-patient variability is shown by the large variance. The comparison of the frequencies of these toxicities between individuals with and without indicated genotypes showed that individuals with MTHFR 677 allele had significantly lower rates of grade 3 leukopenia and individuals with CCND1 A1290 genotype had lower rates of grade 3 leukopenia, grade 2 thrombocytopenia and grade 3/4 liver toxicity, as estimated by increased alanine aminotransferase levels (Table 2). Likewise, when the proportion of weeks with these toxicities was compared between groups with and without the MTHFR TT/CT or CCND1 AA genotype, highly significant results were obtained showing a lower proportion of weeks with the toxicity among individuals with these genotypes. Accordingly, the toxicity rate-ratio associated with the genotype was lower, in most cases showing 2-fold reductions (Table 2). When the combined effect of MTHFR and CCND1 genotypes was assessed, the frequency of weeks with grade 3 leukopenia was further decreased in individuals with both CCND1 AA genotype and MTHFR T677 allele, and increased in individuals without these genotypes (Table 3). There were not significant correlations with the other polymorphisms analyzed.

We previously observed that ALL patients with MTHFR TT/CT or CCND1 AA genotypes tended to have, or, in case of CCND1, had a significantly lower frequency of weeks with MTX dose reduction or withdrawal. However, the average dose of MTX received weekly did not differ across different genotypes of the same polymorphism and was lower than the maximal dose predicted by the protocol leaving the possibility for drug dose adjustment. Here we extended the analysis beyond drug dose by analyzing the relationship between genotypes and the frequency of hematologic and liver toxicity during consolidation and maintenance treatment. Individuals with CCND1 AA or MTHFR
Table 2. The relationship between genotypes and the parameters of high-grade toxicity.

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Descriptive statistics</th>
<th>Genotype</th>
<th>p and RR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MTHFR C1/TT</td>
<td>MTHFR CC</td>
</tr>
<tr>
<td>WBC 3</td>
<td>n. of weeks with toxicity</td>
<td>300</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>median %</td>
<td>1.8</td>
<td>3.5</td>
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<tr>
<td></td>
<td>N. of individuals</td>
<td>44</td>
<td>142</td>
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<tr>
<td></td>
<td>Total n. of weeks</td>
<td>3519</td>
<td>11441</td>
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<tr>
<td></td>
<td>Total n. of weeks with toxicity</td>
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<td>548</td>
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<tr>
<td></td>
<td>median %</td>
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<td>3.1</td>
</tr>
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<td></td>
<td>median %</td>
<td>0.03</td>
<td>0.2</td>
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<tr>
<td></td>
<td>Total n. of weeks with toxicity</td>
<td>74</td>
<td>355</td>
</tr>
<tr>
<td></td>
<td>Median %</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
|          | MTHFR: methylene tetrahydrofolate reductase; CCND1: cyclin D1; WBC 3: grade 3 leucopenia. * total N weeks with toxicity reflects the sum of weeks with grade 3 leucopenia for all patients with indicated genotype. Median % reflects the median frequency of weeks with the given toxicity per patient/per genotype. † median could not be calculated due to the limited number of patients; the mean value is given instead. p1 is obtained by the χ² test and p2 by Mann-Whitney, RR, rate ratio.

TT/CT<sup>677</sup> genotype had lower rates of these toxicities. As for ALL outcome, which was affected more substantially by combined at-risk genotypes than by either genotype alone, grade 3 leucopenia was further decreased in individuals with a combination of the CCND1 AA<sup>10</sup> and MTHFR TT/CT<sup>677</sup> genotypes. Both CCND1 AA<sup>10</sup> and MTHFR T<sup>677</sup> are common among Caucasians, with a frequency of ~40% and ~35%, respectively.<sup>44</sup> The observed correlation for CCND1 follows the pattern predicted from the results of studies associating CCND1 AA<sup>10</sup> genotype with reduced EFS, and is in agreement with the functional impact of this polymorphism. The CCND1 A<sup>10</sup>G substitution modulates the ratio of CCND1 mRNA isoforms. The transcript associated with the CCND1 A allele results in a protein with a longer half-life<sup>7</sup> resembling CCND1 over-expression, which has previously been shown to lead to the increased expression of MTX targets and reduction of sensitivity to MTX.<sup>5</sup>

Reduced MTHFR activity caused by the MTHFR T<sup>677</sup> allele leads to higher 5,10-methylene-tetrahydrofolate levels which, could facilitate uridine-thymidine conversion by TS, reducing the rate of uracil misincorporation into DNA and resulting chromosome damage. This might decrease MTX efficacy explaining both the reduced rates of leukenphia found in this study and the reduced EFS in ALL patients found previously.<sup>19</sup> In agreement with this is the finding showing that cells transfected with T<sup>677</sup> cDNA have decreased MTHFR activity resulting in accelerated cellular growth rate accompanied by decreased chemosensitivity to MTX.<sup>17</sup> Three other studies have addressed the impact of MTHFR on the toxicity in ALL patients. Among adults who underwent treatment with different protocols, doses and schedules of MTX administration, T<sup>677</sup> homozygotes more frequently experienced MTX intolerance although it was not clear how the frequency of the episodes of toxicity were accounted for in the analysis.<sup>11</sup> Kishi et al.,<sup>19</sup> on the other hand found no association between the T<sup>677</sup> allele and either seizures or thrombosis in childhood ALL patients. Likewise, Aplenc et al.<sup>16</sup> did not observe a correlation between MTHFR genotypes and various types of higher-grade toxicity (central nervous system toxicity, diarrhea, hyperbilirubinemia, neuropathy, stomatitis, raised transaminase levels or infection). As in this latter study, we did not observe an influence of MTHFR genotypes on the increase of liver enzyme levels, whereas the other toxicity end-points were not comparable. Chemotherapy toxicity did not differ between carriers of the various genotypes of the other genes analyzed here. The reason for this could be a lower relative importance of some of the genes analyzed. For example, the MTHFD1 variant allele did not play an important role in ALL outcome when analyzed with other prognostic factors.<sup>11</sup> The RFC1 G<sup>0</sup>A polymorphism played a minor role when analyzed simultaneously with other polymorphisms relevant to ALL outcome.<sup>19</sup> On the other hand TS SR homozygosity was clearly associated with poorer ALL outcome after adjustment for other prognostic factors and polymorphisms studied.<sup>19</sup> In this case it is possible that other types of toxicities than those analyzed here...
are different between patients with different TS genotypes. For example, childhood ALL patients with the TS 3R allele were shown to be less prone to osteonecrosis.  

In conclusion, we found that the particular polymorphisms of the folate cycle that correlated with reduced EFS, possibly due to lower sensitivity to MTX, also correlated with lower rates of episodes of toxicity. Although these results should be further verified and the analyses extended to a larger group of patients, this finding opens the possibility of drug dose adjustment: patients who have lower EFS and a lower frequency of chemotherapy toxicity might benefit from an increase in drug dose.

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