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Folate Pathway Polymorphisms Predict Deficits in Attention and Processing Speed after Childhood Leukemia Therapy

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Abstract

Background—Neurocognitive impairment occurs in 20%-40% of childhood acute lymphoblastic leukemia (ALL) survivors, possibly mediated by folate depletion and homocysteine elevation following methotrexate treatment. We evaluated the relationship between folate pathway polymorphisms and neurocognitive impairment after childhood ALL chemotherapy.

Procedure—Seventy-two childhood ALL survivors treated with chemotherapy alone underwent a neurocognitive battery consisting of: Trail Making Tests A (TMTA) and B (TMTB), Grooved Pegboard Test Dominant-Hand and Nondominant-Hand, Digit Span subtest, and Verbal Fluency Test. We performed genotyping for: 10-methylenetetrahydrofolate reductase (*MTHFR* 677C>T and *MTHFR* 1298A>C), serine hydroxymethyltransferase (*SHMT* 1420C>T), methionine synthase (*MS* 2756 A>G), methionine synthase reductase (*MTRR* 66A>G), and thymidylate synthase (*TSER*). Student's two sample t-test and analysis of covariance were used to compare test scores by genotype.

Results—General impairment on the neurocognitive battery was related to *MTHFR* 1298A>C ($p=0.03$) and *MS* 2756A>G ($p=0.05$). Specifically, survivors with *MTHFR* 1298AC/CC genotypes scored, on average, 13 points lower on TMTB than those with *MTHFR* 1298AA genotype ($p=0.001$). The *MS* 2756AA genotype was associated with a 12.2 point lower mean TMTA score, compared to *MS* 2756 AG/GG genotypes ($p=0.01$). The *TSER* 2R/3R and 3R/3R genotypes were associated with an 11.4 point lower mean score on TMTB, compared to the *TSER* 2R/2R genotype

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($p=0.03$). Survivors with >6 folate pathway risk alleles demonstrated a 9.5 point lower mean TMTA score ($p=0.06$) and 14.5 point lower TMTB score ($p=0.002$) than survivors with <6 risk alleles.

Conclusions—Folate pathway polymorphisms are associated with deficits in attention and processing speed after childhood ALL therapy.

MESH keywords

folate; leukemia; neurocognitive; survivor

Introduction

The cure rate for pediatric acute lymphoblastic leukemia (ALL), the most common childhood cancer, is approximately 80%. However, long-term complications of childhood ALL therapy are an emerging health concern for the growing number of survivors in our population. Neurocognitive impairment occurs in 20%-40% of ALL survivors and may result in academic and vocational compromise [1, 2]. Specific deficits occur in attention, processing speed, and memory, which can lead to learning difficulties and declines in global intellect [2, 3]. Cranial irradiation therapy, initially used as central nervous system (CNS) prophylaxis, is clearly associated with neurocognitive impairment, but current treatment protocols have replaced cranial irradiation with intensive systemic and intrathecal chemotherapy in most patients. Methotrexate (MTX) chemotherapy, however, has also been related to neurocognitive deficits by some groups [1, 3, 4] but not others [5, 6]. It seems likely that the degree of neurotoxicity is an interaction of dose, route, and schedule of MTX, as well as timing of leucovorin rescue [7-9], but considerable interindividual variation in neurobehavioral outcomes is observed even in patients treated identically [10].

Demyelinating white matter injury and vascular damage are thought to contribute to the pathogenesis of chronic MTX-related neurotoxicity, but the underlying mechanisms are not well-understood [11, 12]. MTX reversibly inhibits dihydrofolate reductase and leads to reductions in 5-methyltetrahydrofolate (5-MTHF), the primary form of circulating folate and a co-substrate for the remethylation of homocysteine to methionine. Homocysteine and excitatory amino acids subsequently accumulate and may be toxic to the endothelium and sensitize neurons to oxidative damage, ultimately causing neuronal death and diminished myelin synthesis [11-13]. Prior studies have demonstrated decreased 5-MTHF levels and elevated homocysteine in the cerebrospinal fluid (CSF) after MTX therapy [14-16], particularly in patients with acute MTX-related neurotoxicity or leukoencephalopathy [14, 17, 18]. Other neurodegenerative disorders, including severe MTHFR deficiency, idiopathic cerebral folate deficiency, and a few cases of Rett syndrome have also been associated with low 5-MTHF levels or elevated homocysteine levels in the CSF [11, 19-23]. Regardless of etiology, disruption of folate metabolism in the CNS may cause acute or chronic neurological manifestations.

Functional polymorphisms in folate pathway genes may lead to biochemical alterations such as reduced folate levels and elevated homocysteine levels. The following are among the folate pathway genes with functional polymorphisms: 5,10-methylenetetrahydrofolate reductase [*MTHFR* 677C>T (rs1801133) and *MTHFR* 1298A>C (rs1801131)], serine hydroxymethyltransferase [*SHMT* 1420C>T (rs1979277)], methionine synthase [*MS* 2756A>G (rs1805087)], methionine synthase reductase [*MTRR* 66A>G (rs1801394)], and thymidylate synthase in the form of enhancer region repeats (*TSER*). Since folate is critical for CNS development and function, we hypothesized that the presence of certain folate pathway polymorphisms, previously associated with reduced folate or elevated

homocysteine, would be related to neurocognitive deficits after MTX chemotherapy for treatment of ALL. Folate pathway polymorphisms included in the study were selected based on evidence in the literature for functionality of the polymorphisms and a minor allele frequency >5%.

We previously reported that impairment on a brief neurocognitive screen predicted impairment in global intellectual function, reading skills, and mathematical skills in childhood cancer survivors ≥ 6 years old [24]. The screen consisted of a parental questionnaire and standardized clinical performance-based measures, including: Trail Making Test Parts A (TMTA) and B (TMTB) for assessment of attention and processing speed [25], Grooved Pegboard Test Dominant-Hand (PEGDH) and Nondominant-Hand (PEGNDH) for evaluation of fine motor speed [26], Digit Span (DIG) for assessment of working memory [27], and Verbal Fluency Test (CFL) for evaluation of executive function [28]. Impairment on the screen, hereafter referred to as the DIVERGT battery, predicted impaired global intellect ($p<0.0001$). The battery had a sensitivity and specificity for impaired global intellect of 94% and 63% respectively, and it also predicted impairment in reading ($p<0.03$) and mathematics ($p<0.03$) [24].

Krull et al previously reported that ALL survivors with *MTHFR* 1298AC/CC genotypes had a 7.4-fold increased risk of developing attention deficit disorder, as measured by a parental questionnaire [29]. The *MTHFR* 677C>T polymorphism alone was not related to deficits in attention. In the current study, we expanded the panel of folate pathway polymorphisms and evaluated multiple neurocognitive domains, including attention, using the direct child performance measures in the DIVERGT battery. The objective of this study was to further explore whether interindividual variation in folate metabolism influences susceptibility to global neurocognitive impairment and domain-specific impairment in attention, processing speed, and memory after ALL therapy, using direct measures of performance.

Methods

Subjects

Since 2003, long-term survivors at Texas Children's Hospital have been prospectively enrolled on a study protocol to correlate genetic polymorphisms and therapy-related toxicity, including neurocognitive impairment as determined by clinical chart review. The protocol was approved by the institutional review board at Baylor College of Medicine, and survivors (if ≥ 18 years old) or their parents provided informed consent for the study. The recruitment rate is 95%, and participants provide a peripheral blood sample for DNA extraction. As part of routine clinical care, ALL survivors at Texas Children's Hospital are referred for the DIVERGT battery [24]. We identified 72 survivors of B-precursor and T-cell ALL, treated with chemotherapy alone and consecutively enrolled on the above protocol, who had completed the battery and who met eligibility criteria for the current study. Inclusion criteria included age at ALL diagnosis of ≥ 1 year and ≤ 18 years. Exclusion criteria included history of CNS leukemia, cranial irradiation, prior head injury, underlying genetic or neurological disorder associated with neurocognitive impairment, neurodevelopmental disorder associated with folate metabolism (e.g. spina bifida), or pre-existing neurocognitive disorder. Medical charts were abstracted for age at diagnosis, sex, race/ethnicity, leukemia subtype, past medical history, occurrence of early MTX-related neurotoxicity (as defined by acute or subacute onset of seizures, mental status changes, neurologic deficits, or stroke-like encephalopathy), body surface area at diagnosis, number of intrathecal MTX doses, and cumulative intravenous (IV) MTX exposure in g/m^2 of body surface area.

Neurocognitive Evaluation

The DIVERGT battery was conducted prior to genotyping by a trained examiner under the supervision of a neuropsychologist. The tests are age-referenced and nationally standardized to a mean of 100 and a standard deviation of 15. The definition of impairment on the battery has been previously described [24]. Additionally, performance on each of the six measures was evaluated as a continuous variable. A dichotomous variable for domain-specific impairment was also determined for each child performance measure, defined as a score <80 on the individual measure (<10th percentile).

Genotype Analysis

Real-time polymerase chain reaction (PCR) using commercially preformulated TaqMan genotyping assays (Applied Biosystems, Foster City, CA) were used to genotype the *MTHFR* 677C>T (Assay ID C_1202883), *MTHFR* 1298A>C (C_850486_20), *MS* 2756A>G (C_12005959_10), and *MTRR* 66A>G (C_3068176_10) polymorphisms. The 25 μ L reaction mixture included 20 ng genomic DNA, 12.5 μ L of 2 $\times$ TaqMan universal PCR master mix, 1.25 μ L of the respective 20 $\times$ SNP genotyping assay mix, and DNase-free water. The allelic discrimination PCRs were conducted on an Applied Biosystems 7300 Real-Time PCR System using the following conditions: 95°C for 10 minutes and then 35 cycles of: 1) 92°C for 15 seconds and 2) 60°C for 1 minute. For *SHMT* 1420 C>T genotyping, allelic discrimination PCR was conducted similarly using custom-made primers (forward: 5'-CTC CGG GAG GAG GTT GAG A-3'; reverse: 5'-GCC CGC TCC TTT AGA AGT CA-3') and probes (Allele C sequence: 5'-VIC- CTT CGC CTC TCT CTT C-MGB-3'; Allele T sequence: 5'-FAM-TCG CCT CTT TCT TC-MGB-3'). The 25 μ L reaction mixture consisted of: 900 nM each primer, 200 nM each probe, 12.5 μ L of 2 $\times$ TaqMan universal PCR master mix, 20 ng genomic DNA, and DNase-free water. Cycling conditions consisted of 50°C for 2 minutes, 95°C for 10 minutes, and 50 cycles of: 1) 92°C for 30 seconds and 2) 62°C for 1 minute.

The *TSER* polymorphism was evaluated using the standard PCR method described by Hishida et al with modifications [30]. Briefly, 30 ng of genomic DNA were used in a 50 μ L reaction mixture with 0.15 μ M dNTPs, 25 pmol each of forward (5' – CGT GGC TCC TGC GTT TCC – 3') and reverse (5' – GAG CCG GCC ACA GGC AT -3') primers, 5 μ L glycerol, 5 μ L Magnesium-free 10 $\times$ PCR buffer, and 1.5 mM MgCl₂. 0.4 units of Taq polymerase were added to the reaction mixture after a hot-start. Amplification conditions consisted of a 95° denaturation cycle for 10 minutes; 35 cycles of: 1) 95°C for 30 seconds, 2) 61°C for 30 seconds, and 3) 72°C for 30 seconds; and a 72°C final extension cycle for 5 minutes. *TSER* genotypes were ascertained on a 4% agarose gel with ethidium bromide. The 2R/2R genotype was represented by a single 210-bp band, 2R/3R genotype produced 210-bp and 238-bp fragments, and 3R/3R homozygotes had a single 238-bp band. For quality control, 10% of samples were randomly selected for duplicate genotyping for each polymorphism, showing 100% concordance.

Statistical Analysis

For each polymorphism, the genotype related to reduced folate or elevated homocysteine levels in the literature was hypothesized to be the at-risk genotype for neurocognitive dysfunction. Using a dominant model to define exposure, the following were hypothesized to be at-risk genotypes: *MTHFR* 677CT/TT, *MTHFR* 1298AC/CC, *SHMT* 1420CC, *MS* 2756AA, *MTRR* 66AA, and *TSER* 2R/3R or 3R/3R. Outcome measures included impairment on the DIVERGT battery, standard scores on each of the six child performance measures, and domain-specific impairment on each of the six child performance measures. Descriptive statistics were used to summarize study variables. One sample t-test was used to compare the mean score in the study population for each child performance measure to the

expected mean of 100. For univariate analyses, Fisher exact test (categorical variables) and Student's two sample t-test (continuous variables) were used to compare outcomes according to genotype. Potential covariates included age at diagnosis, sex, race/ethnicity, leukemia subtype, number of intrathecal MTX doses, and cumulative IV MTX exposure. High-dose and intermediate-dose IV MTX were included in the computation of cumulative IV MTX exposure. Using median values, dichotomous variables were created for age at diagnosis, number of intrathecal MTX doses, and cumulative IV MTX exposure. Multivariable analyses were performed with logistic regression and analysis of covariance (ANCOVA). All analyses were performed with Stata 10 (Statacorp LP, College Station, TX). A 2-sided p-value of <0.05 was considered significant.

For each participant, we scored the number of adverse alleles (0 to 2) for each polymorphism and then calculated a composite folate pathway genetic risk score (GRS) by summing the number of adverse alleles across the six polymorphisms. The median GRS was used to separate the study population into a high-GRS group (more hypothesized adverse alleles) and low-GRS group (fewer hypothesized adverse alleles). Exploratory analyses were conducted using the methods described above to investigate the relationships between GRS and the primary outcomes.

Results

Study Population

The study population had a male predominance (M:F ratio 2:1) that was slightly higher than expected, but it was otherwise representative of the ALL population seen at Texas Children's Hospital (Table I). Study participants were diagnosed between 1987 and 2001 and treated on various protocols, primarily Pediatric Oncology Group protocols (including POG 9005, POG 9006, POG 9405, POG 9605, POG 8698, and POG 8699). Early MTX-related neurotoxicity occurred in 7 patients (9.7%). Impairment on the DIVERGT battery was observed in 44.3% of the study population. Mean neurocognitive test scores in the overall population demonstrated mild deficits on PEGDH ($p=0.02$), PEGNDH ($p<0.001$) and CFL ($p<0.001$), when compared to the expected means of 100 (Table II). All polymorphisms were in Hardy-Weinberg equilibrium.

Genotype and Impairment on the DIVERGT Battery

Impairment on the battery was related to two polymorphisms: *MTHFR* 1298A>C ($p=0.03$) and *MS* 2756A>G ($p=0.05$). Survivors with the *MTHFR* 1298AC/CC genotypes had a 3.0-fold increased risk of impairment (95% CI 1.1-8.1), compared to those with the *MTHFR* 1298AA genotype. Similarly, those with the *MS* 2756 AA genotype were 3.8 times as likely to meet criteria for impairment on the battery as those with the *MS* 2756AG/GG genotypes. *MTHFR* 677C>T, *SHMT* 1520C>T, *MTRR* 66A>G, and *TSER* polymorphisms were not significantly related to general impairment on the DIVERGT battery.

Genotype and Individual Child Performance Measures

Several genotypes were related to performance on TMTA (focused attention, processing speed) and TMTB (shifting attention, processing speed), as shown in Table III. Survivors with the *MTHFR* 1298AC/CC genotypes performed on average 7 points lower on TMTA ($p=0.11$) and 13 points lower on TMTB ($p=0.001$), compared to those with the *MTHFR* 1298AA genotype. Survivors with the *MS* 2756AA genotype scored on average 12.2 points lower on TMTA ($p=0.01$) and 5.3 points lower on TMTB ($p=0.23$), compared to survivors with the *MS* 2756AG/GG genotypes. Finally, survivors with *TSER* 2R/3R and 3R/3R genotypes scored on average 10 points lower on TMTA ($p=0.07$) and 11.4 points lower on TMTB ($p=0.03$) than those with the *TSER* 2R/2R genotype. *MTHFR* 677C>T, *SHMT*

1420C>T, and *MTRR* 66A>G polymorphisms were not associated with performance on TMTA or TMTB. Complete analyses by genotypes for all six performance measures can be found in Supplemental Table I.

The remaining neurocognitive tests assessed fine motor skills (PEGDH and PEGNDH), working memory (DIG), and executive function (CFL). For survivors with the *MS* 2756AA genotype, the mean PEGNDH score was 11.7 points lower than that of individuals with the *MS* 2756AG/GG genotypes ($p=0.02$). Additionally, survivors with the *MTRR* 66AA genotype scored on average 10.7 points lower on PEGNDH than those with the *MTRR* 66AG/GG genotypes ($p=0.05$). Survivors with the *MTRR* 66AA genotype also scored 10.5 points lower on average on CFL, compared with survivors with the *MTRR* 66AG/GG genotypes ($p=0.01$). Performance on the PEGDH and DIG measures were not associated with any genotype.

We did not observe an association between any child performance measure and any of the following covariates: sex, ethnicity/race, age at diagnosis, leukemia subtype, number of intrathecal MTX doses, and cumulative IV MTX exposure. The associations between genotype and child performance measures that are described above did not change after adjustment for these covariates. Of the 7 survivors with a history of early MTX-related neurotoxicity, 6 (85%) met criteria for long-term neurocognitive impairment on the DIVERGT battery. While early MTX-related neurotoxicity was predictive of long-term neurocognitive impairment ($p=0.03$), 25 of 63 survivors (40%) without early MTX-related neurotoxicity also went on to develop long-term neurocognitive impairment as determined by the DIVERGT battery. The 7 survivors with a history of early MTX-related neurotoxicity scored on average 15.3 points lower on TMTB than survivors without early neurotoxicity ($p=0.03$). Early MTX-related neurotoxicity was not predictive of impairment on TMTA, PEGDH, PEGNDH, DIG, or CFL.

Complete genotyping results for all six polymorphisms were available for computation of the GRS in 59 patients. In this group, the GRS ranged from 0 to 9, with a mean of 6.2 and a median of 6. On average, survivors with a $GRS \geq 6$ scored 9.5 points lower on TMTA ($p=0.06$) and 14.5 points lower on TMTB ($p=0.002$) than survivors with a $GRS < 6$ (Table III). No difference was observed in mean scores on PEGDH, PEGNDH, or DIG between the GRS groups. The association between the GRS and TMTB performance remained significant after adjustment for sex, age at diagnosis, intrathecal MTX exposure, and cumulative IV MTX exposure. We did not observe an association between GRS and impairment on the DIVERGT battery. However, all of the patients who demonstrated impaired performance on TMTA or TMTB (score < 80) had $GRS \geq 6$. No association was observed between GRS and impairment on PEGDH, PEGNDH, DIG, or CFL.

Discussion

This study investigated the association between six folate pathway polymorphisms and neurocognitive deficits in childhood ALL survivors, with the premise that these polymorphisms would modulate neurocognitive function in the setting of intermittent folate depletion due to MTX. In a previous study of 48 childhood ALL survivors, Krull et al reported a 7.4-fold increased risk of attention deficit disorder, as measured by parental questionnaire, in survivors with the *MTHFR* 1298AC/CC genotype [29]. Results of the current study, which includes 24 additional survivors and new independent measures that evaluate child performance directly rather than through a proxy rater, corroborate this earlier finding. Specifically, patients with the *MTHFR* 1298AC/CC genotypes had lower mean scores for direct performance measures of shifting attention and processing speed, namely TMTB. Additionally, the current study provides a more comprehensive investigation of the

role of folate pathway polymorphisms in the development of neurocognitive deficits after ALL therapy. We genotyped a total of six folate pathway polymorphisms in the current study, and our findings suggest that *MS* 2756A>G and *TSER* polymorphisms may also contribute to the interindividual variation in neurocognitive function seen in ALL survivors. The combined effect of multiple polymorphisms on the folate pathway, as measured by the GRS, may be more important than one single polymorphism.

The *MTHFR*, *MS*, and *TS* genes have key functions in the regulation of folate and homocysteine. The *MTHFR* enzyme catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-MTHF [31], and the *MTHFR* 1298CC genotype results in 60% of the usual *MTHFR* enzyme activity [32]. *MS* catalyzes the remethylation of homocysteine to methionine with methylcobalamin as a cofactor [31], and *MS* deficiency results in elevated plasma homocysteine levels [33]. The *MS* 2756A>G polymorphism leads to an amino acid substitution, and the variant G allele has been associated with lower plasma homocysteine levels [33, 34], possibly conferring a protective effect. Finally, *TS* catalyzes the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate [31]. The promoter enhancer region of the *TS* gene may contain two (2R) or three (3R) 28-bp tandem repeat sequences that function as transcriptional enhancer elements. The *TSER* 3R/3R genotype yields higher gene expression levels *in vitro* and higher enzyme activity *in vivo* than the 2R/2R genotype [35]. The 3R/3R genotype is associated with reduced folate and higher homocysteine levels, particularly in individuals with low dietary folate intake [36].

While the *MTHFR* 1298A>C, *MS* 2756A>G, and *TSER* polymorphisms do not directly lead to neurodegenerative disease in normal individuals, they may become critical in the setting of intermittently exaggerated folate depletion caused by MTX therapy for childhood ALL. Alternatively, it is possible that these polymorphisms are not directly associated with neurocognitive dysfunction but are surrogate markers for tightly linked polymorphisms that are in fact responsible for the associations seen in our study. *MTHFR* 1298A>C and *MS* 2756A>G were associated with impairment on the DIVERGT battery, suggesting an impact on global intellectual function. *MTHFR* 1298A>C, *MS* 2756A>G, and *TSER* polymorphisms also appeared to be related specifically to deficits in attention and processing speed, as indicated by TMTA and TMTB. In a study of ALL patients that included patients with cranial irradiation, Krajcinovic et al found that polymorphisms in *MTHFR* 677C>T, *MTHFR* 1298A>C, *MS* 2756A>G, and *MTRR* 66A>G were not related to change in IQ scores over the first four years after diagnosis of ALL. These patients were diagnosed in a similar treatment era as patients on our study but had differences in CNS-directed therapy and did not have long-term neurocognitive evaluations that allow comparison to our study [37].

The combined effect of multiple folate pathway polymorphisms may best predict which children are at risk for treatment-related neurocognitive deficits. Survivors with GRS ≥ 6 performed consistently worse on measures of attention and processing speed and were more likely to demonstrate clinical impairment in these domains. An individual with several at-risk genotypes may have striking variation in folate or homocysteine levels that leads to increased risk for neurocognitive deficits after MTX therapy. As the effects of additional folate pathway polymorphisms are evaluated in a larger sample size, the GRS may be refined to provide a more accurate prediction of which children are at risk for treatment-related toxicity.

Mean scores for TMTA, TMTB, and DIG in our study population were similar to population norms. However, the lack of baseline neurocognitive testing makes it impossible to evaluate whether declines occurred in these domains over time for individual patients. ALL survivors overall scored lower on tests of fine motor speed than expected for age-adjusted population norms. Similar findings have been reported in other studies and have been postulated to be

related to vincristine chemotherapy [38]. Decreased verbal fluency performance has also been previously reported in childhood ALL survivors and do not appear to be related to demographic or treatment variables [39].

One of the strengths of our study was that the study population was drawn from long-term survivors, with median time off-therapy of 4.4 years, ensuring that ample time had elapsed for determination of neurocognitive dysfunction. Additionally, the potential for selection bias is decreased with the use of a validated neurocognitive screening battery, which can be administered in a time-efficient manner during the survivor's annual clinic visit, thereby promoting testing for all ALL survivors.

This study had several important limitations. The study was adequately powered to detect a statistically significant difference on neurocognitive tests of one standard deviation or greater. However, small differences between genotype groups may not have been detected, and we are unable to investigate potential gene-gene or gene-environment interactions. Validation of these results is clearly needed in a larger study. Additionally, the leukemia protocols used to treat participants in this study were heterogeneous and involved some differences in chemotherapy regimens for CNS prophylaxis. These differences between regimens are partially accounted for by inclusion of the important covariates of cumulative IV MTX dose and number of intrathecal MTX doses. We were unable to analyze the dosing schedule of MTX or leucovorin rescue in the current pilot study. Other limitations included a lack of information in this retrospective study on parental education, socioeconomic status, or dietary folate intake, all of which would be important to include in a future study.

Previous studies have shown conflicting results regarding the association between MTX dose intensity and long-term neurocognitive impairment. In a study of 79 survivors of high-risk ALL, neurocognitive performance did not vary between children treated with high-dose MTX and children treated with very high-dose MTX [5]. However, in another study of 36 survivors, those who had received intensified treatment that included high-dose MTX were more likely to have attention deficits than survivors who had received intermediate-dose IV MTX on standard regimens [40]. In our study, we did not observe a relationship between cumulative IV MTX dose or number of intrathecal MTX doses and performance on any of the neurocognitive tests. The study was not designed to investigate this association, so sample size may have been inadequate to detect small differences in test scores among treatment groups. It is also possible, however, that MTX "exposure" from an administered dose, as well as the downstream effects on the folate pathway and neurodevelopment, are moderated by genetic polymorphisms involving folate pathway enzymes. Possible interaction between genotype and MTX exposure will be important to investigate in a larger future study.

Early MTX-related neurotoxicity may predict long-term neurocognitive impairment, but many patients without a history of early MTX-related neurotoxicity also demonstrated long-term neurocognitive deficits. Although this study was not designed to detect an association between the rare outcome of early MTX-related neurotoxicity and folate pathway polymorphisms, further studies may investigate this relationship. Further investigation is also needed to evaluate the association between folate pathway polymorphisms and neurocognitive function in healthy populations. The *MTHFR* 677C>T polymorphism, frequently in the setting of hyperhomocysteinemia, has been associated with vascular dementia, cognitive decline with aging, depression, and schizophrenia in non-cancer populations [41-43]. In a recent study of healthy adolescents, the *MTHFR* 677TT genotype was associated with a mild reduction in cognitive performance when compared to those with the *MTHFR* 677CC/CT genotypes [44]. Further evaluation of the other folate pathway

polymorphisms and neurocognitive outcomes in the general population may help clarify the impact of MTX therapy and possible gene-environment interactions.

In summary, we present additional evidence that folate pathway polymorphisms may modulate the development of neurocognitive deficits in childhood ALL survivors. Future directions include validation of these findings in a larger sample of research subjects and investigation of variation in other folate pathway genes, including cystathione beta synthase, reduced folate carrier 1, and dihydrofolate reductase. Additional pathways that may be important to investigate include other drug metabolism pathways, oxidative stress pathways, and glucocorticoid receptor signaling pathways. Results from these studies may allow us to identify ALL patients at highest risk for neurocognitive impairment and therefore develop interventions to prevent this important late effect of therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I
Characteristics of the Study Population

Characteristic	Study Population			
	n=72	% or Range	Mean	Median
Sex				
Male	48	(66.7)		
Female	24	(33.3)		
Race				
Non-Hispanic Caucasian	42	(58.3)		
Hispanic Caucasian	22	(30.6)		
Other	8	(11.1)		
ALL Subtype				
Standard-risk B-precursor	56	(78.9)		
High-risk B-precursor	12	(16.9)		
T-cell ALL	3	(4.2)		
Age at diagnosis (years)		(1.1 - 10.5)	4.4	3.9
Age at follow-up (years)		(7.2 - 18.5)	12.1	11.3
Time after end of therapy (years)		(0.25 - 12.5)	5.3	4.4
Number of intrathecal MTX doses		(10 - 30)	16.8	16
Mean cumulative IV MTX dose (g/m²)		(1.6 - 30.1)	12	12

Abbreviations: ALL, Acute Lymphoblastic Leukemia; MTX, methotrexate; IV, intravenous; g, gram; m, meter

Table II
Summary of Neurocognitive Test Scores

Neurocognitive test scores	Overall Study Population			
	Mean	SD	Range	% impaired
Total screen	95.4	10.2	(68-118)	44.3
TMTA	100.4	18.1	(49-126)	18.3
TMTB	99.8	17.3	(47-129)	12.9
PEGDH	94.5	19.6	(46-126)	23.6
PEGNDH	90.9	19.6	(46-123)	29.2
DIG	98.9	15.4	(75-135)	5.6
CFL	86.8	14.0	(46-121)	24.3

Abbreviations: SD, Standard Deviation; TMTA, Trail Making Test A; TMTB, Trail Making Test B; PEGDH, Grooved Pegboard Test Dominant Hand; PEGNDH, Grooved Pegboard Test Non-Dominant Hand; DIG, Digit Span; CFL, verbal fluency test

Table III
Mean TMTA and TMTB Scores According to Patient Characteristics

Genotype	n=72	Mean TMTA score	SD	p	Mean TMTB score	SD	p
MTHFR 1298A>C							
AA	35	104.0	13.5	0.11	106.7	12.4	0.001
AC/CC	37	97.0	21.1		93.7	18.8	
MS 2756A>G							
GG/AG	21	109.8	10.3	0.01	105.2	14.0	0.23
AA	40	97.6	19.0		99.9	17.1	
TSER							
2R/2R	14	109.5	10.0	0.07	110.8	10.6	0.03
2R/3R or 3R/3R	43	99.5	19.1		99.4	16.8	
GRS ^a							
<6 risk alleles	17	108.8	9.2	0.06	112.2	7.2	0.002
≥6 risk alleles	42	99.3	19.3		97.7	17.1	

^a Determined for 59 patients with complete genotyping. Abbreviations: TMTA, Trail Making Test A; TMTB, Trail Making Test B; SD, standard deviation; MTHFR, 5,10-methylenetetrahydrofolate reductase; MS, methionine synthase; TSER, thymidylate synthase enhancer region; GRS, genetic risk score