Role of NOS3 DNA Variants in Externalizing Behavioral Problems Observed in Childhood Leukemia Survivors

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Objective: Neuropsychological problems occurrence varies among childhood cancer survivors, and associated risk factors have not been fully deciphered. We wanted to study the role of genetic variants in behavioral problems in this population.

Study Design: Behavioral problems in pediatric acute lymphoblastic leukemia patients (n = 138) were investigated longitudinally, using the Child Behavior Checklist questionnaire and multilevel statistical modeling. Thirty-four candidate polymorphisms, related to anticancer drug effects, were investigated.

Results: NOS3 gene functional polymorphisms showed significant association: patients homozygous for the minor allele at investigated loci showed decreased externalizing behavioral problems scores over time (t-tests: T-786C n = 69, P = 0.003; G894T n = 71, P = 0.065). The effect was even more pronounced for individuals that are homozygous for the −786C844T haplotype (t-test, n = 69, P < 0.001) and results were supported by multilevel modeling analyses (P < 0.001). No such association was observed for internalizing behavioral problems.

Conclusion: NOS3 variants modulate externalizing problems individual trajectories, likely in relationship with glucocorticoid exposure.

Key Words: acute lymphoblastic leukemia, glucocorticoids, pharmacogenetics, Child Behavior Checklist (J Pediatr Hematol Oncol 2013;35:e157–e162)

Behavioral problems may be increased in a significant but transient manner in childhood cancer patients.1,2 We reported that behavioral problems are best predicted by time-circumscribed factors in acute lymphoblastic leukemia (ALL) survivors, such as disease-related variables, and treatment-related pharmacological variables.3 Studies also support a role for specific chemotherapeutic agents in the manifestation of behavioral problems in pediatric cancer survivors, most notably methotrexate (MTX)4–6 and glucocorticoids (GCs).7 Through folate depletion, MTX administration can lead to increased homocysteine (Hcy) levels, which is associated with neurotoxicity.8 Exogenous GC disrupt the hypothalamic-pituitary-adrenal axis response, which can lead to increased risk of mental health-related problems.7

Despite the use of standardized drugs and dosing, treatment-related side effects affect patients differently. Genetic components, particularly polymorphisms associated with drug effects, are likely involved. Given the MTX and GC side effects profile on the nervous system, related genes variants could account for interindividual differences in behavioral problems observed in childhood ALL patients. Methylene tetrahydrofolate reductase, methionine reductase, methionine synthase reductase, endothelial nitric oxide synthase (NOS3), and cystathionine β-synthase (CBS) gene polymorphisms have been found to be associated with variations in Hcy levels.9 The GC receptor (NR3C1) is a key downstream GC effector; NR3C1 variants have been shown to influence exogenous GC therapeutics and side effects.10 Transcriptional activity resulting from NR3C1-GC coupling and binding to glucocorticoid receptor elements is highly regulated11; several additional factors may thus modulate downstream GC-related effects, including nuclear factor-κ-B (NF-κB)12 and NF-κB inhibitor (NFKBIA).13 GC-mediated apoptosis of ALL cells is regulated by several components of the apoptotic machinery, of which Bcl-2 interacting protein Bim and Bcl-2-associated X protein Bax seem to be important contributors.14

Childhood ALL is one of the first cancers for which extensive pharmacogenetic studies have been carried out. Several polymorphisms influencing susceptibility to ALL, survival rates, relapse risk, and treatment-related toxicity have been identified by several groups including ours, as reviewed in.13,14 Despite the considerable potential implications of such studies, there have been few pharmacogenetic studies linking neuropsychological outcomes following anticancer drugs administration. Exceptions are intelligence quotient (IQ) decrease15 and attention disorder16 investigations. Of note, medical treatments, such as exposure to radiation therapy, appeared to interact with the genetic effects.17

Because of their ability to induce neurotoxicity, we postulated that over time, variants of genes responsible for the effects of the anticancer drugs MTX and GC’s effects could affect the prevalence of behavioral problems in patients. This study aimed at testing the role of candidate polymorphisms in a longitudinal follow-up study design on behavioral problems in childhood ALL patients, using a well-validated questionnaire.

MATERIALS AND METHODS

Patient population is composed of 138 patients diagnosed with ALL between 1993 and 1999 (age range, 0 to 18 y)
at CHU Ste-Justine, Montréal, QC, Canada. All patients were treated according to the Dana-Farber Cancer Institute ALL protocol 91-01 or 95-01.19,20 Previous cancer treatment was an exclusion criterion. The majority of patients were of European descents (89.1%). The outcome of interest, patients’ behavioral problems, was evaluated at diagnosis, and 1, 2, 3, and 4 years after diagnosis. At each time point and for a maximum of 5 repeats, mothers completed the Child Behavior Checklist (CBCL), a frequently used and well-validated instrument to investigate behavioral problems in this population.21 divided into global, internalizing (ie, an overcontrol of affects and emotions, such as social withdrawing, somatisation or anxiety/depression), and externalizing ones (ie, an undercontrol of affects and emotions, with difficulties with interpersonal relationships, such as rule-breaking or delinquent behavior, attention problems). CBCL has excellent interinterviewer and test-retest reliabilities, as well as high internal consistency.22 CBCL syndromes also appear as biologically valid as suggested by strong heritability estimates23,24 and biochemical correlations.25,26 These 2 dimensions are conflicting, but not mutually exclusive as some subjects can fluctuate between high levels of externalizing and internalizing problems. Zero, 1, 2, 3, 4, and 5 measurements for the same patient were available for 20, 20, 10, 18, 28, and 42 participants, respectively. Age-standardized and gender-standardized (mean: 50; SD: 10) T scores were used for assessment of problem prevalence and statistical models. Research protocols were reviewed and approved by the CHU Ste-Justine’s Research Ethic Board. Parents’ informed consent was obtained from all participating families.

Genotyping and Polymorphisms
Candidate polymorphisms were mTHFR (A2757G), methionine reductase (A2757G), methionine synthase reductase (A66G), NOS3 (T-786C, G894T), CBS (844 Ins 68), Bax (T-1962G, A-1836T, A-1076G), Bin1 (T-1928G, T-1894C, C298T, A2251T, G2252T), NR3CI (G-3807A, A-627G, G200A, A1220G, C646G, T1511C), NF-κB I (T-1796C), −397 Del, CAAT, A 5 Int 1G, A 6 Int 1G, C 8 Int 23T), NF-κB 2 (−1867 Ins G, C-118T, A-140G, G-26T), NF-κB IA (C-1433T, C-326T, A174G, C1050T). Genotyping details of MTX effect-related polymorphisms have been published previously by our group.17 The same applies for NR3CI polymorphisms G-3807A, A-627G, T1511C, and C646G;27 G200A and A1220G.25 All NF-κB I, 2, IA polymorphisms, as well as Bin1 and Bax gene variations were selected from the National Center for Biotechnology Information database. The frequency of minor allele was not sufficiently high for 7 selected polymorphisms (CBS 844Ins68; Bax A-1836T; NR3CI A-627G/G200A/A1220G/T1511C; NF-κB1T-1796C) to carry on preliminary analyses; these polymorphisms were not further considered. Two polymorphisms (Bax A-1076G; NF-κB IA A174G) were not in Hardy-Weinberg equilibrium (P < 0.05), and were excluded from the analyses, leaving a total of 25 candidate polymorphisms.

Statistical Analyses
Longitudinal data were analyzed through multilevel modeling, in which a level 1 variable modeled time as a dependent variable, and level 2 variables modeled the impact of interindividual specificities on variation in time (initial status and rate of change) as dependent variables.29 Interindividual (level 2) variables were the polymorphisms identified as potential genetic predictors of behavioral problems changes. The major advantage of this statistical method is that it takes into account patients with incomplete set of measurement results in regression parameters estimations, in addition to those of patients with full measurement sets. All, 4, 3, 4, 2, 1, and 0 time points were available for 30.4%, 20.3%, 13.0%, 7.2%, 14.5%, and 14.5% of patients, respectively.

Skewness and kurtosis scores were verified to ascertain normal distribution of continuous variables. These scores calculations and other classic analyses (descriptive statistics, t tests) were performed using SPSS (version 16.0). Multilevel parametric analyses were conducted using SAS MIXED procedure (SAS version 9.2). Linkage disequilibrium (LD) between NOS3 gene variants T-786C and G894T was calculated using Haploview (version 3.32). Validation and P value for LD were obtained by EH (http://linkage.rockefeller.edu/ott/eh.htm) and Phase (version 2.1) was used to infer NOS3 haplotypes.30,31 Office (version 2003) was used for prototype plot production. Given multiple testing, P value was set at 0.002 using Bonferroni correction (0.05/25 candidate polymorphisms). This P value was used in multilevel final model selection; for preliminary analyses and unless otherwise specified, P < 0.05 was considered significant.

RESULTS
Prevalence of Behavioral Problems
On the basis of the reference norms, 16% of the children were expected to have CBCL scores > 60. The proportion of patients in this sample scoring ≥ 60 for global behavioral problems index were 36% and 21%, at diagnosis and 4 years later, respectively. For internalizing problems, proportions of patients above normal score range were 42% (P < 0.001) and 20% (NS), respectively. Regarding externalizing problems at these 2 time points, 20% and 17% of patients were > 60 (both NS), respectively. These findings imply stability of externalizing problems levels despite the significant life changes accompanying the initial treatment period, but showed a transient increased prevalence of internalizing problems during the same period.3

Influence of NOS3 Variants on Externalizing Problems
As preliminary analyses, global CBCL scores were compared for individuals that are homozygous or carriers of minor allele (both heterozygotes and homozygote) for 6 MTX and 19 GC drug effect-related polymorphisms. Analyses were carried out using the latest time point available between 3 and 4 years after diagnosis (data not shown). The only significant difference (P < 0.05) found was when patients were analyzed using NOS3 T-786C polymorphism, more specifically for CC individuals compared to remaining genotypes (t test; n = 80; P = 0.012).

Both internalizing and externalizing problems contribute to the global behavioral score, as genetic variants may impact on each type of behavioral problems, they were further considered separately. No difference was found at any time point between NOS3 T-786CC and remaining genotypes for internalizing problems (NS, data not shown). However, with regard to externalizing problems, CC patients showed significantly lower CBCL scores
4 years later compared to other patients (t test; n = 69; \(P = 0.003\); Fig. 1A). We previously reported that TT carriers for NOS3 G894T polymorphism were more likely to suffer from long-term IQ deficits.\(^{1,2}\) However, with regard to externalizing problems, the difference between homozygous TT individuals and patients with other genotypes for this second NOS3 polymorphism was only found to be only marginally significant at 4 years after diagnosis (t test; n = 71; \(P = 0.065\)) (Fig. 1B).

LD was previously reported between these single-nucleotide polymorphisms (SNPs).\(^{32}\) Our analysis confirmed LD and showed that 4 inferred haplotypes had frequencies compatible with such an association (Table 1; \(P < 0.001\)). A difference for externalizing problems scores between homozygotes for the −786C894T haplotype (arbitrarily named haplotype 2, Table 1) and patients with other haplotypes was statistically significant (t test; n = 69; \(P < 0.001\)) at 4 years after diagnosis (Fig. 1C). The average difference was >10 CBCL points, which is clinically meaningful.

**Multilevel Model of NOS3 Variants as Predictors of Individual Externalizing Problems Trajectory**

To investigate the potential role of homozygosity for NOS3*2 haplotype on individual trajectories of externalizing problems, multilevel modeling was used. Average variation in time of externalizing problems scores was first modeled, excluding any interindividual differences (Base model—Table 2). Parameter estimates indicate that on average, the initial score at diagnosis was 51.71, and that scores decreased of 0.45 points/year after diagnosis. In other words, the best fitted base model taking into account all patients’ scores (\(i\)) at all time point available (\(j\)) is CBCL score \(\mu_{ij} = 51.71 - 0.45(i\times year)\). Four years after diagnosis, the average total decrease was 1.8 points, which bears no clinical significance.

The relationship between the homozygosity for *2 haplotype and individual trajectories was investigated next (*2*2 carriers vs. others—Table 2). The presence of *2*2 had a highly significant impact on the rate of change in CBCL scores. Using stringent Bonferroni corrected \(P\) value of 0.002, individuals homozygous for *2 haplotype showed an average decrease in externalizing problem scores of 3.5 points/year after diagnosis (\(P < 0.001\)), for a total decrease of 14 points after 4 years. Meanwhile, other patients’ scores remained remarkably stable, with a total decrease of only 1.32 points over the same 4-year period \([(-3.50 + 3.17)/year \times 4\) year\]). Prototype plot for the 2 patient groups is shown in Figure 2. As 15 patients were not caucasian, we performed analyses limited to caucasians only and similar results were obtained (impact of *2*2: \(P = 0.003\)). The presence of *2*2, however, did not have any effect on the CBCL initial scores shortly after diagnosis. For individuals with and without *2*2 haplotypes, the initial average scores were 53.81 and 51.69, respectively.

**FIGURE 1.** Average externalizing problem Child Behavior Checklist scores according to patients’ NOS3 genotypes at C-786T (A) and G894T (B) loci, and to *2 haplotype homozygosity (C). **\(P < 0.003\); ***\(P < 0.001\). (A) and (B): patients numbers at 0, 1, 2, 3, and 4 years after diagnosis were 80, 77, 70, 58, and 59 for TT/TC, and 13, 13, 11, 8, 10 for GG/GT, and 10, 11, 11, 7, 10 for TT genotype of G894T polymorphism, respectively. (C): patients numbers for each subgroup, at each time measurement, are indicated in respective columns.
DISCUSSION

Gene variants involved in anticancer drug effects were investigated for their role in behavioral problems in childhood ALL patients over a 4-year period following diagnosis. Despite the stability in externalizing problem scores for the majority of patients, a subset of them carrying 2 minor alleles for NOS3 polymorphisms showed significant decreases in scores over time, as compared to carriers of any other alleles combinations. No such association was found between the investigated polymorphisms and differences in individual trajectories for internalizing problems.

Nitric oxide (NO) acts as a molecular second messenger and is a potent vasodilator. NO production in the nervous system depends on NOS1, but also on NOS3, which is expressed in hippocampal pyramidal cells (p. 76). Altered activity has been reported for NOS3 T-786C and G894T minor variants, resulting in decreased NOS3 gene promoter activity and decreased NO production, respectively. Evidence is suggestive of an association between decreased NOS3 activity and lower level of externalizing problems (ie, improved affect and emotion control). Diminished NOS3 activity was associated with abnormally lower levels of aggression and impulsivity, in humans and in animals. A NOS3 gene haplotype, consisting of 3 functional polymorphisms (including T-786C) was significantly associated with bipolar disorder. In contrast to a previous result, Rujescu et al found a protective effect of NOS3 minor alleles against suicide completion. NOS3−/− mice also show lower levels of aggression, superior spatial learning capabilities, and, in part, antidepressant-like traits compared to wild-type animals. In addition, NOS3−/− mice treated with dexamethasone did not show the increase in NOS activity and showed no NO-mediated vascular protection. In rats, Gulati et al found that NO mimetics have a protective influence against the neurobehavioral alterations and accompanying oxidative injury markers following exposure to emotional stress using the restraint stress paradigm. The common mechanism of the different NOS isoforms could be an effect of neurogenesis, especially in the hippocampus. Together, these findings argue in favor of a contribution of NOS3 minor alleles to decreased externalizing problems.

Consequently, patients with minor alleles for NOS3 polymorphisms would have been expected to have lower levels of externalizing problems scores, compared to others. This was indeed the case, with externalizing problems decreasing with time to lower levels in individuals

TABLE 1. Patients Demographic and NOS3 Polymorphism-related Descriptive Statistics

<table>
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<tr>
<th>Variables</th>
<th>n</th>
<th>X or n (%)</th>
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<td>Age at diagnosis</td>
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<td>0-17</td>
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<td>TT: 42 (32.3)</td>
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<td>Allelic frequency, NOS3 T-786C</td>
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<tr>
<td>Genotype frequency, NOS3 G894T</td>
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<td>GG: 56 (41.8)</td>
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<td>Allelic frequency, NOS3 G894T</td>
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<td>—</td>
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<td>Haplotype frequency</td>
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<td>—</td>
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<tr>
<td></td>
<td></td>
<td>2—CT: 69 (26.5)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3—TG: 133 (51.2)</td>
<td>—</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>4—TT: 21 (8.1)</td>
<td>—</td>
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TABLE 2. Multilevel Modeling of the Impact of *2*2 Homozygosity on Externalizing Problems, from Diagnosis to 4 Years After Diagnosis

<table>
<thead>
<tr>
<th>Fixed Effects</th>
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<td>53.81***</td>
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<td>Intercep</td>
<td>γ01</td>
<td>—</td>
<td>−2.12</td>
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<tr>
<td>Haplotype</td>
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<tr>
<td>Model for β1j</td>
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<td>−3.50***</td>
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<tr>
<td>Slope</td>
<td>γ11</td>
<td>—</td>
<td>3.17***</td>
</tr>
<tr>
<td>Haplotype</td>
<td><em>2</em>2</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

***P < 0.001.
Patients were coded “0” if *2*2, “1” otherwise.

**FIGURE 2.** Prototype plot illustrating the change in rate of externalizing problems during the study period in individuals with and without *2*2 haplotype.
homzygous for −786C. We already demonstrated that externalizing problems tend to decrease more steeply over the 4 years of follow-up in ALL patients who received a more potent GC treatment (dexamethasone compared to prednisone) in the first 2 years of treatment, which demonstrates the effects of GC exposure on externalizing problems in this cohort. According to this linear model, minor allele NOS3 carriers showed a steeply descending slope of externalizing behavior scores over the 4 years of follow-up. This decrease with time is likely to reflect a return to the baseline level after treatment termination, from a higher level during treatment, likely related to GC exposure.

We also found NOS3 T−786C and G894T SNPs to be in LD. This has also been reported in a German study, suggesting complementary effects of minor alleles.

We have previously identified that homozygous individuals for the minor T allele of G894T polymorphism were at higher risk for long-term IQ decline in this population. Moreover, impact differences of G894T variants on IQ decline were exacerbated when adjusting for whether brain irradiation was included as part of the treatment. However, the impact of this variant was only marginal with respect to behavioral problems, and its interaction effect with brain irradiation was not significant (data not shown). Finally, haplotype *4 (−786T 894T), rather than haplotype *2, was associated with neurocognitive decline (Maja Krajnovic, unpublished data, 2011). Genetic liabilities to cognitive and behavioral problems thus appear distinct even when analyzed within the same gene. Globally, MTX-related Hey level modulation was suspected to be the main neurotoxicity factor when investigating neurocognitive outcomes, whereas GC are more likely to have an effect on behavior.

The limited sample size is a limitation to this study. Some other of the 25 investigated polymorphisms associations may have been left undetected given lack of statistical power. These findings should be replicated as sampling effects might also account for random association.

To our knowledge, this is the first study to report that NOS3 genetic specificities might have an impact on the behavior in a pediatric population; a finding reported so far in adult patients only. Evidence from this study and others support an important role for NO in normal and abnormal development of CNS, and provide additional cues in understanding chemotheraphy-induced neurotoxicity, and in designing future intervention strategies for the management of externalizing problems in pediatric oncology patients.

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REFERENCES