Expression and polymorphism (rs4880) of mitochondrial superoxide dismutase (SOD2) and asparaginase induced hepatotoxicity in adult patients with acute lymphoblastic leukemia

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Asparaginase, which depletes asparagine and glutamine, activates amino-acid stress response. Oxidative stress mediated by excessive reactive oxygen species (ROS) causes enhanced mitochondrial permeabilization and subsequent cell apoptosis and is considered as a plausible mechanism for drug-induced hepatotoxicity, a common toxicity of asparaginase in adults with acute lymphoblastic leukemia (ALL). Studies investigating the pharmacogenetics of asparaginase in ALL are limited and focused on asparaginase-induced allergic reaction common in pediatric patients. Here, we sought to determine a potential association between the variant rs4880 in SOD2 gene, a key mitochondrial enzyme that protects cells against ROS, and hepatotoxicity during asparaginase-based therapy in 224 patients enrolled on CALGB-10102, a treatment trial for adults with ALL. We report that the CC genotype of rs4880 is associated with increased hepatotoxicity following asparaginase-based treatment. Thus, rs4880 likely contributes to asparaginase-induced hepatotoxicity, and functional studies investigating this single-nucleotide polymorphism (SNP) are needed to develop therapeutic approaches that mitigate this toxicity.

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INTRODUCTION

The intensive use of asparaginase is an essential component of pediatric regimens for acute lymphoblastic leukemia (ALL) and has been associated with significant improvements in survival. Accordingly, many attempts have incorporated asparaginase in treatment regimens for adults with ALL; yet, the higher rate of asparaginase-related toxicities in adults with ALL have limited its widespread use and new insights are needed. Studies investigating the pharmacogenetics of asparaginase in ALL are limited and mostly focused on pediatric patients with ALL. A recent study investigated more than 500 000 single-nucleotide polymorphisms (SNPs) in 485 children with ALL and found an association of 5 SNPs in the GRIA1 gene with hypersensitivity to the drug. The same group also tested more than 2 million SNPs using the HapMap lymphoblastoid cell lines and identified the aspartate metabolic route as the most likely candidate pathway for asparaginase sensitivity. In addition, polymorphisms in genes that mediate the antileukemic effect of asparaginase, such as the asparaginase synthetase gene, the basic region leucine zipper activating transcription factor 5 and the argininosuccinate synthase 1 gene, were found to be associated with reduced event-free survival of childhood patients with ALL, but not with toxicity.

Although asparaginase allergy is the main toxicity observed in children, hepatotoxicity is one of the most common toxicities of this drug in adults with ALL and often limits the use of this effective drug in this age group. The incidence rate of elevated liver enzymes and hyperbilirubinemia (grade 3 or 4) was reported to be 36 and 14%, respectively, in adults compared with 20 and 3% in pediatric patients. Studies that focused on exploring these toxicities in association with polymorphisms in adult ALL are still limited. Superoxide dismutase (SOD), an enzyme that catalyzes the dismutation of superoxide (O2•−) into oxygen and hydrogen peroxide, is a crucial antioxidant that protects cells against oxidative stress. Three forms of SOD enzymes are present in mammalian cells: cytoplasmic superoxide dismutase (SOD1), mitochondrial superoxide dismutase (SOD2) and extracellular superoxide dismutase (SOD3). Two previous studies have reported that the SOD2 polymorphism causing a V16A amino-acid substitution (rs4880) is significantly associated with drug-induced liver injury (DILI). Recently, the same polymorphism was found to be significantly correlated with breast cancer survival after cyclophosphamide-containing chemotherapy.

Here, we studied 224 patients enrolled on CALGB-10102, a treatment trial for adults with previously untreated ALL who received L-asparaginase as part of their chemotherapy regimen. The aim of this study is to investigate potential associations between the SOD2 rs4880 polymorphism and asparaginase-related hepatotoxicity in adult patients with ALL. Secondary objectives of this study are to assess a possible correlation between this polymorphism and ALL susceptibility in adults, and to determine whether this polymorphism is associated with SOD2 transcript levels as a possible mechanism for this functional...
variant. Here, we also genotyped rs4958351 in the GRIA1 gene, one of the SNPs that was previously identified to be associated with hypersensitivity to asparaginase in children with ALL.2

MATERIALS AND METHODS
Patient population
We studied samples obtained from 224 patients with previously untreated ALL, enrolled on a national clinical trial for adults with ALL (Cancer and Leukemia Group B [CALGB] trial 10102). Informed consent to use the tissue for investigational studies was obtained from each patient enrolled on the trial and according to institutional guidelines. Complete clinical data were available for 221 of 224 patients. Samples at remission (after Cycle III of treatment) were available from 196 patients for DNA extraction and genotyping. Paired samples of pretreatment and post-remission peripheral blood (bone marrow paired samples were obtained from two patients) samples from 30 patients were available for RNA analysis. Remission samples (after Cycle III of treatment) obtained from 86 patients were used for RNA analysis and correlation with hepatotoxicity and genotypes (Supplementary Table S1).

By Cycle III of the treatment regimen on the CALGB-10102 protocol, patients would have received the following chemotherapy: Cyclophosphamide, Daunorubicin, Vincristine, i-asparaginase, Cytarabine, Methotrexate, and 6-Mercaptopurine. i-asparaginase was given as 6000 U/m² SC or IM twice a week for six doses beginning on day 1 during the first month of treatment and on days 15, 18, and 22 during the second and fourth month of treatment. Common Terminology Criteria for Adverse Events v4.0 (CTCAE) was used to grade hepatotoxicity.

RNA extraction and real-time quantitative PCR
Total RNA was isolated from bone marrow and peripheral blood samples using Trizol reagent (Life Technologies Carlsbad, CA, USA). cDNA was synthesized using SuperScript III reagents (Life Technologies) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed using commercially available TaqMan Gene Expression Assay primers and probes for SOD2 and B2M and the LightCycler 480II Real-Time PCR System (Roche, Basel, Switzerland). The expression levels were normalized to B2M gene expression.

DNA extraction and genotyping
Genotyping was performed using the TaqMan Allelic Discrimination Assay for rs4880 of SOD2 gene and rs4958351 in the GRIA1 gene (Life Technologies). The PCR was carried out using The LightCycler 480 System (Roche). After PCR, fluorescence from reaction products was measured and analyzed using the System LightCycler 480 Software (Roche, Basel, Switzerland).

Statistical analysis
We tested agreement with the Hardy–Weinberg Equilibrium using a χ2 goodness-of-fit test for rs4880. Because 84% of patients with ALL in our study reported to be white, we used genotyping data obtained in European cohorts, after excluding patients of Hispanics origin (N = 16). We then compared genotype frequencies of the polymorphisms between cases with ALL and reported frequencies obtained from the NCBI European cohort data sets (ESP cohort, Hap-Map-CEU and CEU-GENO). We also compared genotype frequencies in Hispanic patients with ALL and Hispanic/Latino/Mexican-American cohort data set (HSP-genos-panel). We used the Fisher’s Exact test, and calculated odds ratios (ORs) with 95 percent confidence intervals (95% CI). In the association study, P-values of less than 0.05 were considered to be statistically significant. Expression levels of SOD2 transcripts between three or two groups were tested with the Kruskal–Wallis test or an unpaired Mann–Whitney test, respectively. The Chi-square test was then used to compare differences in allele frequencies and genotype distribution of the polymorphism between patients with hepatotoxicity and those without. The Fisher’s Exact test was used to test the recessive model.

RESULTS
Patient population and hepatotoxicity data
We studied samples obtained from 224 patients with ALL. Demographic and clinical data were available for 221 patients (age range, 17–80 years; median age, 43.9 years; 85 female and 136 male), enrolled on treatment trial (CALGB protocol 10102) for adults with ALL. Asparaginase hepatotoxicity was estimated by assessing the following clinical laboratory markers: aspartate transaminase (AST), alanine transaminase (ALT), albumin, alkaline phosphatase and bilirubin levels in patients following induction and two cycles of post-remission therapy. Among the 221 patients, 51 (23%) patients had grade 3 or 4 elevated AST levels, and 82 (37%) patients had grade 3 or 4 elevated ALT levels and 53 (24%) patients had grade 3 or 4 elevated bilirubin levels. AST and ALT levels may be increased several folds above normal in hepatocyte injury. On the other hand, elevations of alkaline phosphatase and bilirubin levels predominate in cholestatic syndromes.10 Therefore, we defined hepatotoxicity as grade 3/4 of both AST and ALT, grade 4 of either AST or ALT, or grade 3/4 of bilirubin elevation. Using this classification, we identified 73 patients (33.03%) meeting these criteria (Table 1).

Genotyping analysis for SOD2 rs4880 in patients with ALL
DNA samples from 196 patients with ALL were genotyped for SOD2 rs4880 and GRIA1 rs4958351. Among these, four samples were excluded from the SOD2 rs4880 analysis due to poor discrimination/quality of genotyping results. The genotypes of the remaining 192 samples were used for further analysis. Our genotyping results showed a minor allelic frequency of 0.52 (for the C allele). Among the 192 patients, 48 patients had a TT genotype (25%), 55 patients had a CC genotype (28.6%) and 89 patients had a CT genotype (46%). These results did not deviate from the Hardy–Weinberg equilibrium (Chi-square (χ²) test P = 0.32 with 1 degree of freedom).

We compared genotype frequencies of SOD2 rs4880 obtained from our data set of patients with ALL, with publicly available databases. Because 84% of the patients enrolled on CALGB-10102 were Caucasian, we compared our results of SOD2 rs4880 genotype frequencies obtained from 192 patients with data from public data bases of European cohorts. We found that the frequency of the CC genotype (28.6%) in our data set was significantly higher than that (14.8%) of the CEU-GENO (two-tailed Fisher’s Exact test; P = 0.007, Table 2). Higher frequency of the CC genotype was also found in the population of patients with ALL when compared with that in the ESP cohort (P = 0.029) or the HapMap-CEU cohort (P = 0.14; Table 2, Supplementary Table S2). Since the rs4880 CC genotype is more frequent in the Hispanic population, we reanalyzed the samples after excluding patients of Hispanic ethnicity (N = 16). We found that the frequency of the CC genotype remained higher in patients with ALL in comparison with the CEU-GENO panel (P = 0.049) and a trend for higher frequency was observed when we compared our data with that of

| Hepatotoxicity data | Number of patients (N) | Percent of patients (%) |
|---------------------|------------------------|-------------------------|
| Total               | 221                    | 100                     |
| AST grade 3/4       | 51                     | 23.08                   |
| ALT grade 3/4       | 82                     | 37.10                   |
| Albumin grade 3      | 29                     | 13.12                   |
| ALK-Pho grade 3/4    | 30                     | 13.57                   |
| Bilirubin grade 3/4  | 53                     | 23.98                   |
| Hepatic failure grade 4/5 | 5               | 2.26                     |
| ALT and AST grade 3, either grade 4 or bilirubin grade 3/4 | 73 | 33.03 |

Abbreviations: ALL, acute lymphoblastic leukemia; ALT, alanine transaminase; AST, aspartate transaminase.

Table 1. Hepatotoxicity rates in 221 adult patients with ALL received asparaginase-based treatment
the ESP or Hap-Map-CEU cohorts. In patients with ALL of Hispanics origin in comparison with that in the HSP-GENO cohort ($P = 0.165$). On the other hand, among the 196 patients genotyped for GRIA1 rs4958351 there were 21 or 10.7% AA; 86 or 43.8% AG; and 89 or 45.4% GG. The minor allelic frequency was 0.33, and the genotyping results did not deviate from the Hardy–Weinberg equilibrium (Chi-square ($\chi^2$) test $P = 0.97$ with 1 degree of freedom). The observed frequency distribution was similar to that of the Hap-Map-CEU population ($P = 0.98$, Supplementary Table S3).

Association of SOD2 polymorphism rs4880 and hepatotoxicity

We analyzed 221 patients for whom hepatotoxicity data were available; among them we obtained genotyping data on 190 patients for SOD2 rs4880 and 193 patients for GRIA1 rs4958351. Two and three patients with genotyping data but no hepatotoxicity data were excluded from the analysis of rs4880 and rs4958351, respectively. The correlation between the rs4880 genotype and hepatotoxicity parameters is shown in Table 3. Grade 3 and 4 of bilirubinemia was found more frequently in patients with the SOD2 rs4880 CC genotype compared with those with the CT genotype (Fishier's exact test $P = 0.055$). In addition, the SOD2 rs4880 CC genotype was associated with a trend toward more serious (Grade 4) hepatotoxicity compared with the rs4880 CT or TT genotypes ($P = 0.09$). Albumin levels did not correlate significantly with rs4880 genotype.

As previously described, we defined hepatotoxicity as grade 3/4 of both AST and ALT, grade 4 of either AST or ALT, or grade 3/4 bilirubin elevation. Using this classification, we identified 61 of the 190 patients (32%) meeting these criteria. We tested the possible genetic models (dominant, recessive or additive effect). We found that only the CC genotype was associated with increased risk of hepatotoxicity (Chi-square test $P = 0.018$, OR $= 2.6$, 95% CI 1.1–6.07; $P = 0.026$ when CC vs TT were compared, OR $= 2.5$, 95% CI 1.2–5.1; $P = 0.01$ when CC vs CT were compared) suggesting a recessive model. Therefore, we implemented the recessive model for further analysis and found that patients with the SOD2 rs4880 CC genotype had a significantly higher frequency of hepatotoxicity than those with the TT or CT genotypes (Fishier's exact test $P = 0.006$; OR $= 2.53$, 95% CI 1.3–4.8; Table 3 and Figures 1a and b) The Pharmacogenomics Journal (2017), 108 (including Hispanics) 0.016 0.049 0.165

Patients with ALL (excluding Hispanics) 176 0.267 0.466 0.267

Hispanics patients with ALL 16 0.50 0.43 0.06

Abbreviations: ALL, acute lymphoblastic leukemia; SNP, single-nucleotide polymorphism. aThe Chi-square with Yates’ correction was calculated instead of Fisher’s exact test, due to the large number of samples.

Table 3. The rs4880 SOD2 SNP genotype frequencies in adult patients with ALL and association with hepatotoxicity

| Genotype                      | Total N (%) | CC, N (%) | CT, N (%) | TT, N (%) | Chi-square test P-value (recessive model) |
|-------------------------------|-------------|-----------|-----------|-----------|-------------------------------------|
| Total N (%)                   | 190 (100)   | 55 (28.9) | 88 (46.3) | 47 (24.7) | 0.069                                |
| AST grade 3/4                 | 42 (22.1)   | 16 (38.1) | 21 (50)   | 5 (11.9)  | 0.18                                 |
| ALT grade 3/4                 | 68 (35.7)   | 19 (27.9) | 30 (44.1) | 19 (27.9) | 0.86                                 |
| Albumin grade 3/4             | 26 (13.7)   | 9 (34.6)  | 10 (38.4) | 7 (26.9)  | 0.49                                 |
| ALK-phos grade 3/4            | 27 (14.2)   | 10 (37)   | 9 (33.3)  | 8 (29.6)  | 0.36                                 |
| Bilirubin* grade 3/4          | 43 (22.6)   | 18 (41.8) | 14 (32.5) | 11 (25.6) | 0.05                                 |
| Any Hepatotoxicity grade 4/S  | 23 (12.1)   | 10 (43.4) | 10 (43.4) | 3 (13)    | 0.09                                 |
| Both AST and ALT grade 3 or either AST or ALT grade 4 Or Bilirubin 3/4 | 61 (32.1) | 26 (42.6) | 23 (37.7) | 12 (19.6) | 0.006 |

Abbreviations: ALL, acute lymphoblastic leukemia; ALT, alanine transaminase; AST, aspartate transaminase. aBased on total and direct bilirubin measurements.

bThree patients had hepatic failure of grade 5.

Table 2. The rs4880 SOD2 SNP genotype frequencies in adult patients with ALL and comparison with its frequencies in European and Hispanics cohorts

| Cohort                   | Total (N) | CC (%) | CT (%) | TT (%) | Chi-square vs CT vs TT analysis | Chi-square vs CT+TT analysis |
|--------------------------|-----------|--------|--------|--------|-------------------------------|-----------------------------|
|                          |           |        |        |        | P-value | P-value | P-value | P-value |
|                          |           |        |        |        | (including Hispanics) | (excluding Hispanics) | Hispanics | (including Hispanics) | (excluding Hispanics) |
| ESP Cohort               | 4368      | 0.207  | 0.504  | 0.288  | 0.146  | 0.35  | 0.16  | 0.01*  | 0.069  |
| Hap-Map-CEU              | 226       | 0.221  | 0.451  | 0.327  | 0.14  | 0.35  | 0.16  | 0.01*  | 0.069  |
| CEU-GENO                 | 108       | 0.148  | 0.5    | 0.352  | 0.016  | 0.049 | 0.165 | 0.007  | 0.019  |
| HSP-GENO                 | 108       | 0.333  | 0.389  | 0.278  | 0.016  | 0.049 | 0.165 | 0.007  | 0.019  |
| Patients with ALL (excluding Hispanics) | 176 | 0.267  | 0.466  | 0.267  | 0.016  | 0.049 | 0.165 | 0.007  | 0.019  |
| Hispanics patients with ALL | 16 | 0.50   | 0.43   | 0.06   | 0.016  | 0.049 | 0.165 | 0.007  | 0.019  |

Abbreviations: ALL, acute lymphoblastic leukemia; ALT, alanine transaminase; AST, aspartate transaminase. A Based on total and direct bilirubin measurements.
**SOD2 mRNA expression in patients with ALL**

Next we sought to examine SOD2 mRNA levels in cells obtained from patients with ALL, assess changes in SOD2 mRNA levels following treatment, and correlate these levels with hepatotoxicity. SOD2 mRNA expression was measured by RT-PCR in samples obtained from patients with ALL (N = 86) that completed induction therapy and two post-remission cycles on CALGB-10102. SOD2 mRNA levels were not significantly different between patients with hepatotoxicity, and those without (Figure 2a). Patients were dichotomized into SOD2 high expressers and SOD2 low expressers, using the median expression as the cutoff. No significant correlation was observed when we assessed each toxicity parameter, or toxicity with grade 3 and higher.

In those patients for whom we had paired samples (pretreatment and post-remission, N = 30), we analyzed changes in SOD2 mRNA expression. Interestingly, SOD2 mRNA expression was significantly lower in pretreatment samples in comparison with remission samples (Figure 2b). Although rs4880 is known to affect the mitochondrial translocation of SOD2, in order to exclude other possible transcriptional regulatory mechanisms for this SNP, we examined the expression of SOD2 mRNA in pretreatment (N = 25) and remission (N = 86) samples relative to their rs4880 genotype. We did not observe significant correlation between SOD2 mRNA levels before, or during treatment and rs4880 genotypes (Figures 3a and b).

**DISCUSSION**

Asparaginase, which depletes asparagine and glutamine, activates an amino-acid stress response. Similar to other cytotoxic agents, the anti-cancer activity is associated with oxidative stress, which is mediated by excessive reactive oxygen species (ROS), resulting in elevated mitochondrial permeabilization and subsequent cell apoptosis.11,12 This process is thought to represent a common mechanism of drug-induced hepatotoxicity. High ROS levels resulting from variability in the function or expression of enzymes involved in these pathways may affect therapeutic outcomes and toxicities.13

SOD2 is located predominantly in the mitochondrial matrix and has an important role in the detoxification of mitochondrial superoxide14,15 by converting superoxide into hydrogen peroxide and oxygen.16 This enzyme is indispensable for cell survival; while, homozygous SOD2-deficient mice die shortly after birth,17 heterozygous SOD2-deficient mice are viable but present with increased susceptibility to chemical-induced mitochondrial toxicity in the liver.18–20 This suggests an important role of this gene in protecting the liver from drug-induced toxicities.

The polymorphism rs4880 in SOD2 results in the incorporation of either alanine (C allele) or valine (T allele) in the mitochondrial targeting sequence of the protein. The alanine form of SOD2 is transported normally into the mitochondria, while the protein with valine is partially trapped in the inner mitochondrial membrane.21 Although this suggests that the T allele would be associated with lower enzymatic efficiency, higher levels of ROS and greater risk of cancer and toxicities, most pharmacogenomic correlative studies have implicated the C allele as a risk allele for...
cancer.\textsuperscript{22–24} Here, we reveal that the CC genotype, but not CT or TT, is likely present at higher frequencies in patients with ALL. We also found this genotype to be more frequent in the Hispanic cohort and Hispanics are known to have a higher incidence of ALL compared with other ethnicities.\textsuperscript{25} Polymorphisms in SOD2 have not previously been associated with susceptibility to ALL; perhaps, the lack of large pharmacogenomics studies in adult patients with ALL may have previously limited the statistical power to identify this locus.

Importantly, in this study we found that the CC genotype was associated with asparaginase-related hepatotoxicity in adult patients with ALL. Patients in the study have also received methotrexate and 6-Mercaptopurine; both drugs may contribute to asparaginase-associated hepatotoxicity. Current clinical guidelines recommend holding asparaginase treatment in adults when grade 3–4 hepatotoxicity develops and then re-challenging with careful monitoring if toxicity resolves to grade 1, often resulting in prolonged delays in treatment and suboptimal dosing which may impair treatment outcomes.\textsuperscript{1} Therefore, the findings resulting from our pharmacogenomic approach may have important clinical implications.

Interestingly, SOD2 transcript levels were significantly higher in samples obtained at remission compared with those obtained at diagnosis. This may be attributed to the different cell composition, being leukemic cells in the diagnosis samples and normal cells in the remission samples. Asparaginase-induced SOD2 mRNA upregulation may also contribute to this finding. The transcript levels of SOD2 did not correlate with this rs4880 polymorphism, or differ between patients with and without hepatotoxicity. Previous studies have shown that this variant affected the enzymatic activity but not the transcript level of SOD2. SOD2 activity has been reported to be 33\% higher in CT or TT individuals compared with CC individuals.\textsuperscript{26} Although we recognize that our study is limited by the relatively small number of patients analyzed and the lack of a control cohort, our data suggest that genetic variation in the SOD2 gene is associated with susceptibility to ALL in this adult cohort and is associated with treatment-related hepatic toxicities; thus, a larger cohort is required to validate these findings. Furthermore, functional studies that investigate this genetic association are needed in order to develop therapeutic approaches that might mitigate this toxicity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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