Glutathione S-transferase M1 polymorphism: a risk factor for hepatic venoocclusive disease in bone marrow transplantation

Alok Srivastava, Balasubramanian Poonkuzhal, Ramachandran V. Shaji, Biju George, Vikram Mathews, Mammen Chandy, and Rajagopal Krishnamoorthy

Hepatic venoocclusive disease (HVOD) in bone marrow transplantation (BMT) is attributed to toxicity of cytoreductive agents, especially busulfan and cyclophosphamide, in the conditioning therapy. Busulfan, as well as the metabolites of cyclophosphamide, are conjugated with glutathione (GSH), catalyzed by enzymes of the glutathione-S-transferase (GST) family. To assess the impact of polymorphisms of the GST genes, GSTM1 and GSTT1, on the risk of HVOD, we evaluated 114 consecutive patients with β-thalassemia major undergoing BMT. There was a significantly increased incidence of HVOD in patients with the GSTM1-null genotype compared with those with the GSTM1-positive genotype (46.5% vs 18.3%; P = .001). Pharmacokinetic analysis in these patients showed that the clearance of busulfan was higher and first-dose steady-state concentration was lower among those with HVOD (0.403 ± 0.06 vs 0.33 ± 0.071 L/h/kg, Student t test P value = .0001; and 508 ± 125 vs 656 ± 255 ng/mL, t test P value = .001, respectively). We conclude that the GSTM1-null genotype predisposes to HVOD, and the sinusoidal endothelial cells and hepatocyte damage may be mediated by metabolites of busulfan through depletion of the cellular GSH pool. (Blood. 2004;104:1574-1577)

© 2004 by The American Society of Hematology

From the Department of Haematology, Christian Medical College, Vellore, India; and Institut National de la Sante et de la Recherche Medicale (INSERM) U 458, Hopital Robert Debre, Paris, France.


Supported in part by the Indo-French Center for the Promotion of Advanced Research (IFCPAR), project no. 2403-2.

A.S. and B.P. contributed equally to this study.

Reprints: Alok Srivastava, Department of Haematology, Christian Medical College, Vellore 632 004, India; e-mail: aloks@cmcvellore.ac.in.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology
GSTM1 POLYMORPHISM: RISK FACTOR FOR HVOD

Diagnosis of HVOD and analysis of potential contributing factors

HVOD was diagnosed based on Baltimore criteria\(^\text{20}\) as follows: development of hyperbilirubinemia with serum bilirubin level greater than 34.2 μM (2 mg/dL) with any 2 of the symptoms including ascites, painful hepatomegaly, and unexplained weight gain of more than 5% from baseline within 20 days of BMT. Liver biopsies were not done for diagnosis of HVOD but the liver was examined at autopsy in 2 patients who succumbed to HVOD. Weight was recorded twice a day and liver function tests were assessed at least twice a week in all patients. Age, serum bilirubin level, ferritin level, weight, and hepatitis B or C virus infection, and Lucarelli et al.'s\(^\text{21}\) classification of patients were correlated with the incidence of HVOD.

Busulfan assay and pharmacokinetic analysis

Bu levels in plasma samples were analyzed by a high-performance liquid chromatography (HPLC) method using derivatization with tetrafluoroboric anhydride as previously reported.\(^\text{22}\) Pharmacokinetic analysis was done using the TOPFIT program (Gustav Fischer, Stuttgart, Germany)\(^\text{23}\) as described previously.\(^\text{18}\) Bu doses were not adjusted to achieve any predetermined target level.

Assay of hepatic GSH

Glutathione levels in pre-BMT liver biopsy tissue homogenates were measured by HPLC with fluorescent detection as described previously.\(^\text{24}\) Glutathione levels are expressed as nmol/mg protein.

Analysis of the GSTM1/T1 polymorphism

Genomic DNA from peripheral blood from all patients collected before BMT and from 250 healthy controls was subjected to a multiplex polymerase chain reaction (PCR) procedure reported earlier\(^\text{25}\) for simultaneous amplification of the GSTM1 and GSTT1 genes using albumin as the internal control. The genotype assignment was made by electrophoretic analysis of the amplified product in 2% agarose gel. This technique clearly identifies the homozygous null genotype but does not discriminate the deletional heterozygotes from nondeletional homozygotes, both of which were classified as GSTM1-positive genotype.

Statistical analysis

Statistical significance of the difference between groups was calculated by chi-square test, Fischer exact test, or Student t test as applicable. Crude odds ratio (OR) was calculated with 95% confidence intervals. All statistical analyses were performed using Epi Info statistical program (Centers for Disease Control and Prevention, Atlanta, GA)\(^\text{26}\) and SPSS version 7.5 (Chicago, IL) for Windows. Multivariate logistic regression analysis was done for the possible predictors of HVOD as independent variables.

Results

A total of 114 patients with β-thalassemia major underwent BMT during the period of this study. Their median age was 6 years (range, 2-16 years). Pretransplantation risk stratification by Lucarelli et al.'s\(^\text{21}\) classification placed 9 patients in class I, 49 in class II, and 56 in class III. The maximum weight gain and the highest bilirubin level in the first 20 days after BMT are shown in Table 1. All patients with HVOD had hepatomegaly more than 2 cm.

The maximum bilirubin level and the percentage weight gain before day +20 was significantly higher in patients with HVOD than those without HVOD (116.96 ± 77.29 μM vs 20.86 ± 8.55 μM [6.84 ± 4.52 mg/dL vs 1.22 ± 0.5 mg/dL]; P = .00001 and 14.2% ± 4.95% vs 3.6% ± 2.1%; P = .00001, respectively). Pretransplantation bilirubin levels were similar between these 2 groups. There was a significantly higher incidence of HVOD in patients with the GSTM1-null genotype compared with those with the GSTM1-positive genotype (46.5% vs 18.3%; P = .001; Table 2). There was no significant difference in the incidence of HVOD in patients with GSTT1-null or -positive genotype (18% vs 33.5%; P = .13).

Of the 33 patients with HVOD, 8 had severe disease. Seven of them died with HVOD as the major cause of death. Six (75%) of 8 of these patients had the GSTM1-null genotype. The GSTT1-null genotype was seen in 6 (18%) of 33 patients with HVOD against 28 (35%) of 81 patients without HVOD.

The mean hepatic GSH levels in the pretransplantation liver biopsy homogenates of 58 patients for whom the data were available showed no significant difference between those with or without HVOD (48.7 ± 36 vs 40 ± 35 nmol/mg protein; P = .38, respectively).

The results of univariate and multivariate analysis of the known risk factors of HVOD are given in Table 3. Of all the variables that appeared significant on univariate analysis, only age and GSTM1 genotype turned out to be significant predictors of HVOD on multivariate analysis.

The clearance (Cl/F) and steady-state concentration (Css) of oral Bu after the first dose was significantly higher in thalassemic patients who developed HVOD compared with those who did not develop HVOD (0.403 ± 0.06 vs 0.33 ± 0.071 L/h/kg; P = .00001 and 508 ± 125 vs 656 ± 255 ng/mL; P = .001, respectively). When Bu Cl/F was included as one of the variables in multivariate analysis, patients with a Cl/F above 0.37 L/h/kg had a significantly higher risk of developing HVOD compared with those with a Cl/F below 0.37 L/h/kg (OR, 4.1; P = .02). Further, patients with the GSTM1-null genotype showed significantly higher Cl/F and lower Css of Bu after the first dose compared with those with the GSTM1-positive genotype (Table 4).

Table 1. Comparison of weight gain and bilirubin levels after BMT in patients with and without HVOD

<table>
<thead>
<tr>
<th></th>
<th>Maximum weight gain, % (range)</th>
<th>Maximum bilirubin level, μM (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With HVOD</td>
<td>14.24 ± 4.95 (6.3-27.5)</td>
<td>116.96 ± 116.45 (37.62-506.16)</td>
</tr>
<tr>
<td>Without HVOD</td>
<td>4.67 ± 3.8 (0-9.3)</td>
<td>21.03 ± 9.41 (8.55-30.78)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;.00001</td>
<td>0.00001</td>
</tr>
</tbody>
</table>

The maximum weight gain and the highest bilirubin level in the first 20 days after BMT are shown. The conversion factor for serum bilirubin level is as follows: mg/dL × 17.1 = μM.

<table>
<thead>
<tr>
<th></th>
<th>GSTM1 null, n (%)</th>
<th>GSTM1 positive, n (%)</th>
<th>GSTT1 null, n (%)</th>
<th>GSTT1 positive, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVOD+</td>
<td>23 (53.5)</td>
<td>58 (81.7)</td>
<td>28 (82)</td>
<td>53 (66.5)</td>
</tr>
<tr>
<td>HVOD−</td>
<td>20 (46.5)*</td>
<td>13 (18.3)*</td>
<td>6 (18)†</td>
<td>27 (33.5)†</td>
</tr>
</tbody>
</table>

Numbers in each group are as follows: GSTM1 null, n = 43; GSTM1 positive, n = 71; GSTT1 null, n = 34; and GSTT1 positive, n = 80.

*P = .001 by chi-square test.
†P = .13 by chi-square test.
Cl/F-1, clearance after the fi

Various pretransplantation and transplantation-related risk factors have been implicated in the pathogenesis of HVOD. However, no single factor has so far been shown to be independently significant. Our study in a large uniform cohort of patients demonstrated that prior administration of Bu significantly altered exposure to Bu and Cy. The observed increase in the incidence of HVOD in patients with lower Bu area under the concentration versus time curve (AUC) and hence higher Bu clearance (Cl/F-1) and residual steady-state concentration (C.Css-1) is consistent with previous findings that prior administration of Bu significantly altered exposure to Cy. The observed increase in the incidence of HVOD in the GSTM1-null patients may be due to altered metabolism of these drugs, generating toxic metabolites that result in increased risk of HVOD.

Multivariate logistic regression analysis of all the potential risk factors of HVOD (age, serum bilirubin level, ferritin level, AST level, ALT level, hepatitis B and C virus infections, and Lucarelli et al’s classification of patients) revealed age and the GSTM1-null genotype as the only significant predictors of HVOD (OR, 0.832; P = .02; OR, 4.12; P = .0011). Age has previously been shown to be a risk factor. Our data highlights the importance of GSTM1-null status as a new and additional risk factor for HVOD.

To further explore the potential mechanisms that could explain the association of the GSTM1 polymorphism with the elevated risk of developing HVOD, we examined the correlation between Bu pharmacokinetic parameters and HVOD. We found that the Cl/F of oral Bu was significantly higher in thalassemic patients who developed HVOD compared with those who did not develop HVOD. Patients with Cl/F above 0.37 L/h/kg had a significantly higher risk of developing HVOD compared with those below this level (OR, 4.1; P = .02). Patients with the GSTM1-null genotype showed significantly higher Cl/F and lower first-dose Css of Bu compared with those with the GSTM1-positive genotype (Table 4). This is also consistent with our earlier observation of significantly increased incidence of HVOD in patients with lower Bu area under the concentration versus time curve (AUC) and hence higher Bu Cl/F. Based on these data, it is likely that HVOD results from hepatocyte/SEC damage due to the metabolite of Bu (rather than Bu itself) either directly or indirectly through depletion of the cellular GSH pool. In fact, it has been suggested by DeLeve et al. that hepatic toxicity of Bu could be due to either GSH depletion or the GSH-conjugated metabolite of Bu, depending on the GSH status of the cell. We did not find any difference in the pre-BMT hepatic GSH levels in the liver in 58 patients with and without HVOD for whom the data were available. It may be more appropriate to assess postconditioning SEC/hepatic GSH levels and their relationship with hepatic toxicity possibly in other models. It is of interest to note that SECs are more vulnerable to GSH-depleting drugs than hepatocytes, the latter being more efficient in synthesizing GSH than SECs.

For Bu Cl/F to be higher, elevated levels of GSTA1 would be expected, as this is the major isoenzyme involved in the metabolism of Bu. We have shown a 2- to 4-fold increase in the enzyme and mRNA levels of GSTA1 in patients with β-thalassemia major with the GSTM1-null genotype compared with those with the GSTM1-positive genotype. This would result in enhanced metabolism of Bu and GSH depletion, thereby causing increased toxicity in these patients. It has also been reported by Ritter et al. that elevated expression levels of GSTA1-1 in patients treated with

| Table 3. Pre-BMT characteristics of patients and their influence on the incidence of HVOD |
|---------------------------------|-----------------|----------------|----------------|----------------|----------------|
| With HVOD                        | No HVOD         | OR             | 95% CI         | P              |
| Age, y, mean ± SD                | 9.58 ± 4.6      | 6.52 ± 3.8     | 0.844          | 0.76-0.94      | .0014          |
| Serum bilirubin level, µM, mean ± SD | 20.18 ± 11.97     | 15.05 ± 7.7    | 0.38           | 0.179-0.803    | .0112          |
| Serum ferritin, ng/mL, mean ± SD | 3799 ± 1728     | 3400 ± 2095    | 0.999          | 0.99-1.0       | .334           |
| AST, U/L, mean ± SD              | 98 ± 74         | 61 ± 28        | 0.984          | 0.97-0.99      | .0014          |
| ALT, U/L, mean ± SD              | 132 ± 102       | 77 ± 56        | 0.99           | 0.98-0.99      | .0016          |
| Lucarelli, n                     | 2              | 7              | 1.0            | NA             | NA             |
| Class I                         | 10             | 39             | 2.1            | 0.39-11        | .38            |
| Class II                        | 21             | 35             | 2.34           | 0.97-5.6       | .058           |
| Class III                       | 8              | 4              | 6.16           | 1.7-22.2       | .0055          |
| Hepatitis B/C infection, n       | 20             | 23             | 4.12           | 1.7-6.67       | .0011          |
| GSTM1-null genotype, n           | 0.421          | 0.155-1.14     | .0884          | 0.563          | 0.163-1.94     | .362          |

The conversion factor for serum bilirubin level is as follows: mg/dL × 17.1 = µM.
NA indicates not applicable.

| Table 4. Busulfan pharmacokinetic parameters with reference to GSTM1 genotype |
|---------------------------------|-----------------|----------------|----------------|
|                                | GSTM1 null      | GSTM1 positive | GSTM1 null     |
|                                | 544 ± 184       | 667 ± 256      | 0.40 ± 0.064   |
|                                | 0.001           | 0.333 ± 0.071  | 0.00001        |

C.Css-1 indicates steady-state concentration after the first dose of busulfan; and Cl/F-1, clearance after the first dose of busulfan.

*P-value calculated by 2-tailed t-test.
Bu could accelerates tissue factor expression due to injury to the SECs, shifting these patients to a procoagulative state. They postulated that variability in the expression of GST-α could explain the unpredictable occurrence of HVOD. Nevertheless, we cannot exclude the possibility that the GST-μ enzyme encoded by the GSTM1 locus could play a major role in hepatocytes/SEC protection, and its absence in individuals with the null genotype could be detrimental to these cells.

McDonald et al.\(^{10}\) have recently shown a association between the AUC of Cy and its metabolites and the incidence of hepatic toxicity. Our data clearly suggests that Bu or its metabolites cause hepatic toxicity that may be worsened by subsequent exposure to Cy, resulting in HVOD. It would be important to evaluate the impact of Bu and Cy individually and sequentially on hepatic toxicity, and its absence in individuals with the null genotype could be detrimental to these cells.

The overall frequencies of the GSTM1- and GSTT1-null genotypes in these patients were not significantly different from those seen in the healthy controls (37.7% vs 33% had the GSTM1-null genotype, 29% vs 26% had the GSTT1-null genotype, and 9.6% and 8% had both null genotypes among 114 thalassemic patients and 250 healthy controls, respectively), quite similar to those reported for Asian Indian population,\(^{31,32}\) and the frequency of the GSTM1-null genotype is lower than that has been reported for another Caucasian population (33% vs 50%; \(P = .003).^{16}\) Hence, the high incidence of the GSTM1-null genotype in thalassemic patients with HVOD cannot be attributed to increased frequency of this genotype in the Indian population.

We conclude that the GSTM1-null genotype is an independent risk factor for HVOD in patients with β-thalassemia major undergoing BMT. Its identification prior to BMT could allow individualization of therapy with regard to conditioning regime and prophylaxis for HVOD.

Acknowledgment

We would like to acknowledge the help provided by Ms M. L. Kavitha in statistical analysis.

References

32. Garte S, Gaspari L, Alexandrie A-K, et al. Muta-
Glutathione S-transferase M1 polymorphism: a risk factor for hepatic venoocclusive disease in bone marrow transplantation

Alok Srivastava, Balasubramanian Poonkuzhali, Ramachandran V. Shaji, Biju George, Vikram Mathews, Mammen Chandy and Rajagopal Krishnamoorthy