Effect of the GSTM1 Null Genotype on Glutathione S-Transferase (GST) Activity in Patients with Non-Viral Liver Tumors

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Abstract: Glutathione S-transferase (GST) is a major phase II drug-metabolizing enzyme. Several isoforms of human GST as well as different GST genetic polymorphisms are known, but limited data exists concerning the relationship between GST polymorphisms and GST activity using 1-chloro-2,4-dinitrobenzene in human liver. To resolve this query, we analyzed the genetic polymorphisms of four main GST isoforms [GST mu 1 (GSTM1), GST theta 1 (GSTT1), GST alpha 1 (GSTA1), GST pi 1 (GSTP1)] and measured hepatic GST activity isolated from the same patients. We found that GSTM1 null individuals have significantly lower (P = 0.0082) GST activity compared with GSTM1 positive individuals. No significant changes in GST activity were observed in individuals with GSTT1, GSTA1, and GSTP1 genotypes. Interestingly, the levels of GST activity exhibited were similar when compared with GSTA1*A/A and GSTA1*A*/B, and GSTP1*A*/A and GSTP1*A*/B, respectively, if the genotype was GSTM1 null. Therefore, the genotypes of GSTA1*A*/B and GSTP1*A*/B individuals do not significantly affect the level of hepatic GST activity. An examination of the correlation between GST mRNA expression and GST activity subsequently revealed a significant correlation between GSTM1 mRNA levels and GST activity (r = 0.626, P = 0.007). These data are expected to facilitate research on the prediction of efficacy and safety of GSTM1 null-mediated drug metabolism and may establish whether genetic polymorphisms of the GST gene, specifically GSTM1, can act as a biomarker.

Key words: glutathione S-transferase (GST), gene polymorphisms, human liver, non-viral liver carcinoma, drug metabolism

Introduction

Glutathione S-transferase (GST) consists of multiple gene products that catalyze the conjugation of a wide variety of electrophilic drugs and chemicals. Cytosolic GST isozymes comprise seven different isoforms; alpha (A), mu (M), omega (O), pi (P), sigma (S), theta (T) and zeta (Z)1). The genetic polymorphisms of GSTM, GSTT, GSTA and GSTP influence...
the efficacy and safety of anti-cancer drugs, such as oxaliplatin, and affect cancer risk\textsuperscript{2–4}. Previous studies of the genetic polymorphisms associated with GST isoforms have established that the genetic differences in GSTM1, GSTT1, GSTA1 and GSTP1 decrease GST activity\textsuperscript{1, 2}. GSTM1 null (gene deletion)\textsuperscript{3}, GSTT1 null (gene deletion)\textsuperscript{4}, \(GSTAI^*B\ (−567T>G + −69C>T + −52G>A)\textsuperscript{7}\), and \(GSTPI^*B\ (exon 5, 313A>G)\textsuperscript{8}\) are the major polymorphisms that affect GST activity. Zhou \textit{et al} have reported that GSTM1 null [formally known as GST-\(\mu\) (negative)] decreases GST activity when compared with GST-\(\mu\) (positive)\textsuperscript{9}. On the other hand, Arakawa \textit{et al} have recently revealed that the GSTM1 null genotype does not alter GST activity\textsuperscript{10}. GST plays an important role in the detoxification of many electrophilic compounds with reduced glutathione in the liver. Unfortunately, there is no reliable data on the relationship between GST variants and GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in the human liver.

In the present study, we focused on four GST isoforms (GSTM1, T1, A1 and P1) and compared the relationship between genetic variants of GST isoforms and GST activity using CDNB.

\textbf{Materials and methods}

\textit{Patients}

Biopsy specimens of normal human liver (noncancerous liver tissues) were obtained from 18 Japanese hepatectomized patients (12 men, six women) with non-viral liver carcinoma (hepatocellular carcinoma, three patients; intrahepatic cholangiocarcinoma, six patients; metastatic liver cancer from colorectum and/or gastric cancer, nine patients) at Showa University Hospital. The liver specimens were numbered anonymously. Written informed consent was obtained from all patients before the operation. This study was conducted in accordance with the Declaration of Helsinki and its amendments and was approved by Human Genome Ethics Committee, Showa University. Genomic DNA and total RNA were isolated from 18 individual liver samples but because insufficient cytosolic material was obtained in one specimen a total of 17 specimens were used.

\textit{Genotyping}

Genomic DNA was extracted from liver tissue using a NucleoSpin\textsuperscript{®} Tissue Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Genotyping of \(GSTM1\) and \(GSTT1\) null individuals was performed using multiplex polymerase chain reaction (PCR)\textsuperscript{11}. \(GSTAI^*B\) (promoter region, \(-567T>G + −69C>T + −52G>A\)) was detected using the primers as described by Coles \textit{et al}\textsuperscript{7}. \(GSTPI^*B\) (exon 5, 313A>G) was detected using the primers (5’-tccccagtgactgtgttg-3’ and 5’-gaagccctttctttgttca-3’) designed by the Primer3 output program (http://bioinfo.ut.ee/primer3-0.4.0/). \(GSTAI^*B\) and \(GSTPI^*B\) were identified by direct sequence methods as described by Toda \textit{et al}\textsuperscript{12}. PCR conditions and methods for the analysis of the GST genotype were performed as described by Kashiwabara \textit{et al}\textsuperscript{13} with some modification. The PCR mixture contained 1.0 to 2.5 U of AmpliTaq\textsuperscript{®} Gold DNA polymerase (Life
Technologies Japan Co., Ltd., Tokyo, Japan) and 150 to 300 ng genomic DNA. The annealing
temperatures ranged from 53°C to 64°C. Direct sequences were performed by BigDye®
Terminator methods using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies
Japan Co., Ltd.). Reaction products were purified and then dried under vacuum. All samples
were resuspended with 25 µl of Hi-Di™ formamide (Life Technologies Japan Co., Ltd.) and
analyzed on an ABI310 or ABI3100 Genetic Analyzer (Life Technologies Japan Co., Ltd.).

Total RNA Isolation and Real-Time PCR

Total RNA was isolated from liver tissues using the acid guanidinium thiocyanate-phenol-
chloroform extraction method14. Total RNA yield, purity and integrity were determined by the
\( A_{260}/A_{280} \) absorbance ratio (>1.6) and confirmed by examination of the RNA by electrophoresis
on 1.2% agarose/formamide gels. Reverse transcription was performed using a TaKaRa RNA
LA PCR™ Kit (AMV) Ver.1.1 (TaKaRa Bio Co., Ltd., Shiga, Japan). Real-time PCR was
performed on an Eppendorf Mastercycler® RealPlex2 (Eppendorf Japan Co., Ltd., Tokyo,
Japan) using the TaqMan® Gene Expression Assay (Life Technologies Japan Co., Ltd.) for
GSTM1 (Hs01683722_gH), GSTT1 (Hs00184475_m1), GSTA1 (Hs00275575_m1) and GSTP1
(Hs02512067_s1), and the TaqMan® Gene Expression Master Mix (Life Technologies Japan Co.,
Ltd.). Constitutively expressed human *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH)
(Hs00266705_g1) was used as an internal control for normalization.

Cytosol Extraction

Liver tissues were homogenized with five volumes of 1.15% KCl solution using a Potter-
Elvehjem homogenizer. The hepatic cell cytosol was isolated by differential centrifugation and
the cytosolic samples were immediately stored at −80°C. Protein concentration was estimated by
the method of Lowry et al15 using bovine serum albumin as a standard.

GST Activity Measurement and Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Sigma-Aldrich Co. Ltd. (Tokyo,
Japan). GST activity was measured by the method of Habig et al16 using CDNB as a
substrate. The final concentration of CDNB in a reaction mixture was 1 mM. A unit of GST
activity was expressed as µmol/mg protein/min.

Statistical Analysis

Primary analysis was performed by Mann-Whitney U test for GST activity categorized
into GST genotypes. \( P \) values were calculated for two-sided tests of primary analysis, with
Bonferroni correction \( P < 0.0083 \) considered statistically significant. As a secondary analysis,
Spearman’s rank correlation coefficient was used, and the relationship between mRNA expression
and GST activity was evaluated. Data were analyzed with SPSS Statistics Version 23.0 (IBM
Japan, Co., Ltd., Tokyo, Japan).
Results and discussion

Initially, we analyzed the genetic variants associated with different GST isoforms. The genotype frequencies of GSTM1, GSTT1, GSTA1 and GSTP1 were determined and the frequencies of GSTM1 null, GSTT1 null, GSTA1*A/B and GSTP1*A/B genotypes were 77.8%, 33.3%, 22.2% and 38.9%, respectively (Table 1). The data was compared with published data and the Japanese Single Nucleotide Polymorphisms (JSNP) database (http://snp.ims.u-tokyo.ac.jp/index.html) and we observed a higher frequency of GSTM1 null genotype in this study (77.8%) than in Munaka et al (49.3%) \(^{17}\). These data suggest that GSTM1 null may be a useful biomarker in patients with non-viral liver carcinoma.

Fig. 1 shows the relationships between GST activity and GST genotypes [GSTMI (A), GSTT1 (B), GSTA1 (C), and GSTPI (D)]. GST activity is significantly decreased in the GSTM1 null genotype in comparison with GSTM1 positive (Fig. 1A, \(P = 0.0082\)). This observation agrees with the findings of Seidegård et al\(^5\). The GSTT1 null, GSTA1*B, and GSTPI*B genotypes have been observed previously to affect GST activity\(^6\-8\). We did not observe any statistically significant difference between GST activity and genotypes (Fig. 1B, C, D), indicating that liver GST activity is affected mainly by GSTM1 variant.

On the basis of these findings, we investigated whether the GSTM1 null genotype affects GST activity when the sample has the GSTA1*A/B and GSTP1*A/B or GSTP1*A/B and GSTPI

<table>
<thead>
<tr>
<th>GST Genotypes</th>
<th>Number of patients and frequency</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
</tr>
<tr>
<td>Null</td>
<td>14</td>
</tr>
<tr>
<td>GSTT1</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
</tr>
<tr>
<td>Null</td>
<td>6</td>
</tr>
<tr>
<td>GSTA1</td>
<td></td>
</tr>
<tr>
<td>*A/*A</td>
<td>14</td>
</tr>
<tr>
<td>*A/*B</td>
<td>4</td>
</tr>
<tr>
<td>GSTP1</td>
<td></td>
</tr>
<tr>
<td>*A/*A</td>
<td>11</td>
</tr>
<tr>
<td>*A/*B</td>
<td>7</td>
</tr>
</tbody>
</table>

GSTA1*A and GSTP1*A are wild-type genotypes. GSTA1*B and GSTPI*B have polymorphisms of the GST promoter region (−567T>G, −69C>T, −52G>A) and Ile\(^{105}\)Val, respectively. The number in parentheses indicates genotype frequencies; n is the number of patients.
GST Genotypes and Activity in Human Liver

A

B

C

D

E

F

Fig. 1. Relationship between GST activity and four GST isoform genotypes (GSTM1, GSTT1, GSTP1, GSTA1; A–D) in human liver (n = 17). Panels E and F show the relationship between GST activity and GSTA1 (E, *A/*A and *A/*B) or GSTP1 (F, *A/*A and *A/*B) genotypes carried with GSTM1 null genotype (n = 14). The horizontal bar denotes the median. The boxes represent the distribution of 25% to 75% and the whiskers show the lowest and highest. GST activity values were analyzed by the Mann-Whitney U test (**P < 0.01); n is the number of patients. 1-Chloro-2,4-dinitrobenzene was used to measure GST activity.

*A/*B genotypes. GSTA1 and GSTM1 are major GST isoforms and are apparently expressed in human liver. There were no statistically significant differences in GST activity between GSTA1*A/*A and GSTA1*A/*B or GSTP1*A/*A and GSTP1*A/*B (Fig. 1E, F). Similar results have been reported by Zhou et al in patients with hepatitis B virus (HBV) infection.
Although the patients in our study had no HBV infection, we concluded that GSTM1 null is a major genotype affecting GST activity when using CDNB as substrate in human liver. In addition, the GSTM1 null gene may be useful as a biomarker for the prediction of low GST activity.

Recently, Arakawa et al reported that the GSTM1 null genotype does not affect GST activity when using CDNB as a substrate. Their results suggest that GSTA1 and GSTP1 may affect GST activity. We investigated and compared GST activity between GST genotypes including GSTA1*A/B and GSTP1*B because CDNB is a general substrate used to detect the total catalytic activity of GSTM1, GSTA1 and GSTP1. However, we did not observe any differences in activity among the GST isoforms, which indicates that GSTM1 null genotype has a critical role in GST expression and in the maintenance of GST activity in human liver.

The correlation between GST mRNA expression and GST activity is shown in Fig. 2. The Spearman's rank correlation coefficients between GSTM1, GSTT1, GSTA1 and GSTP1 mRNA levels and GST activity exhibited \( r = 0.626 \) (\( P = 0.007 \)), \( r = 0.187 \) (\( P = 0.473 \)), \( r = -0.167 \) (\( P = 0.523 \)), and \( r = -0.172 \) (\( P = 0.510 \)) in GSTM1, GSTT1, GSTA1 and GSTP1, respectively. Thus, we observed a significant correlation between GSTM1 mRNA expression and GST activity.

GSTM1 and GSTA1 mRNAs are expressed predominantly in the liver. Therefore, it is possible that GSTA1 affects GST activity but we did not observe a decrease in GST activity in the GSTA1*A/B genotype (Fig. 1C, E). In this respect, Arakawa et al showed that GST activity is apparently maintained with GSTM1 null genotype when compared with GSTM1 positive genotype. In addition, the GSTA1*B genotype has been shown to result in decreased protein expression and GST activity because of decreased activation of the proximal promoters of GSTA1 in HepG2 cells. The aryl hydrocarbon receptor (AhR) regulates the expression of GSTA1 and GSTA2 mRNAs, therefore we investigated the relationship between the expression of AhR mRNA and GSTA1 mRNA. There is no significant difference between the expression levels of these genes (\( r = 0.195 \), data not shown), which suggests that AhR does not regulate GSTA1 mRNA expression.

Moscow et al and Nishimura and Naito have reported that GSTP1 mRNA is expressed at a low level in the liver in comparison with other tissues. In the present study, we observed that GSTP1 mRNA is expressed in human liver. GSTP1 mRNA expression is significantly induced by the presence of the GSTP1 Val variant allele. Therefore, it is apparent that GSTP1 genotypes affect GST activity. Although it has been reported that GSTA1*B as well as the GSTP1*B genotypes could significantly affect GST activity, we observed that GSTA1*B has little effect on GST activity, indicating that GSTM1 genotypes may be largely responsible for GST activity.

In conclusion, we investigated the simultaneous analysis of GST genotypes including GSTM1, GSTT1, GSTA1 and GSTP1 and liver GST activity in Japanese patients with non-viral liver tumors. Although we obtained a limited number of patient specimens, we identified that the GSTM1 positive or GSTM1 null genotypes are responsible for GST activity using CDNB as a substrate. A single GST substrate, CDNB, was used in this study because this compound
is a common substrate for measurement and evaluation of the enzymatic activities of GSTM1, GSTA1 and GSTP1 in human liver\textsuperscript{21}. A number of factors are associated with the regulation of GST activity and inter-individual differences have been reported. The results of this study will facilitate the prediction of the efficacy and safety of GST-mediated drug metabolism including platinum-containing anti-cancer agents such as cisplatin and oxaliplatin. This study indicated that genetic polymorphisms of the GST gene can act as a biomarker, however studies with a greater number of participants are necessary to clarify the relationship between the genotype of GST isoforms and GST activity.
Acknowledgements

The authors thank Misato Shimizu for technical assistance in part of this study.
This work was supported in part by Grants-in-Aid for Private University High Technology Research Center Project matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology).

Conflict of interest disclosure

The authors have declared no conflict of interest.

References


[Received January 6, 2016: Accepted January 15, 2016]