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Original

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

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the efficacy and safety of anti-cancer drugs, such as oxaliplatin, and affect cancer risk[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[1, 2]. *GSTM1* null (gene deletion)[5], *GSTT1* null (gene deletion)[6], *GSTA1*B* (−567T>G + −69C>T + −52G>A)[7], and *GSTP1*B* (exon 5, 313A>G)[8] are the major polymorphisms that affect GST activity. Zhou *et al* have reported that *GSTM1* null [formally known as GST-μ (negative)] decreases GST activity when compared with GST-μ (positive)[9]. On the other hand, Arakawa *et al* have recently revealed that the *GSTM1* null genotype does not alter GST activity[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.

Materials and methods

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.

Genotyping

Genomic DNA was extracted from liver tissue using a NucleoSpin® Tissue Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Genotyping of *GSTM1* and *GSTT1* null individuals was performed using multiplex polymerase chain reaction (PCR)[11]. *GSTA1*B* (promoter region, −567T>G + −69C>T + −52G>A) was detected using the primers as described by Coles *et al*[7]. *GSTP1*B* (exon 5, 313A>G) was detected using the primers (5'-tccccagtgactgtgtgttg-3' and 5'-gaagccccttttctttgttca-3') designed by the Primer3 output program (http://bioinfo.ut.ee/primer3-0.4.0/). *GSTA1*B* and *GSTP1*B* were identified by direct sequence methods as described by Toda *et al*[12]. PCR conditions and methods for the analysis of the *GST* genotype were performed as described by Kashiwabara *et al*[13] with some modification. The PCR mixture contained 1.0 to 2.5 U of Ampli*Taq*® Gold DNA polymerase (Life

Technologies Japan Co., Ltd., Tokyo, Japan) and 150 to 300 ng genomic DNA. The annealing temperatures ranged from 53℃ to 64℃. 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 method[14]. 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® RealPlex^2 (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℃. Protein concentration was estimated by the method of Lowry *et al*[15] 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 al*[16] 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).

Table 1. Frequencies of *GST* genotypes in Japanese patients with non-viral liver carcinoma

GST Genotypes	Number of patients and frequency	
	n	%
GSTM1		
Positive	4	(22.2)
Null	14	(77.8)
GSTT1		
Positive	12	(66.7)
Null	6	(33.3)
GSTA1		
$^*A/^*A$	14	(77.8)
$^*A/^*B$	4	(22.2)
GSTP1		
$^*A/^*A$	11	(61.1)
$^*A/^*B$	7	(38.9)

GSTA1**A* and *GSTP1***A* are wild-type genotypes. *GSTA1***B* and *GSTP1***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.

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[17, 18] 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 [*GSTM1* (A), *GSTT1* (B), *GSTA1* (C), and *GSTP1* (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 *GSTP1***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*/**A* and *GSTA1***A*/**B* or *GSTP1***A*/**A* and *GSTP1*

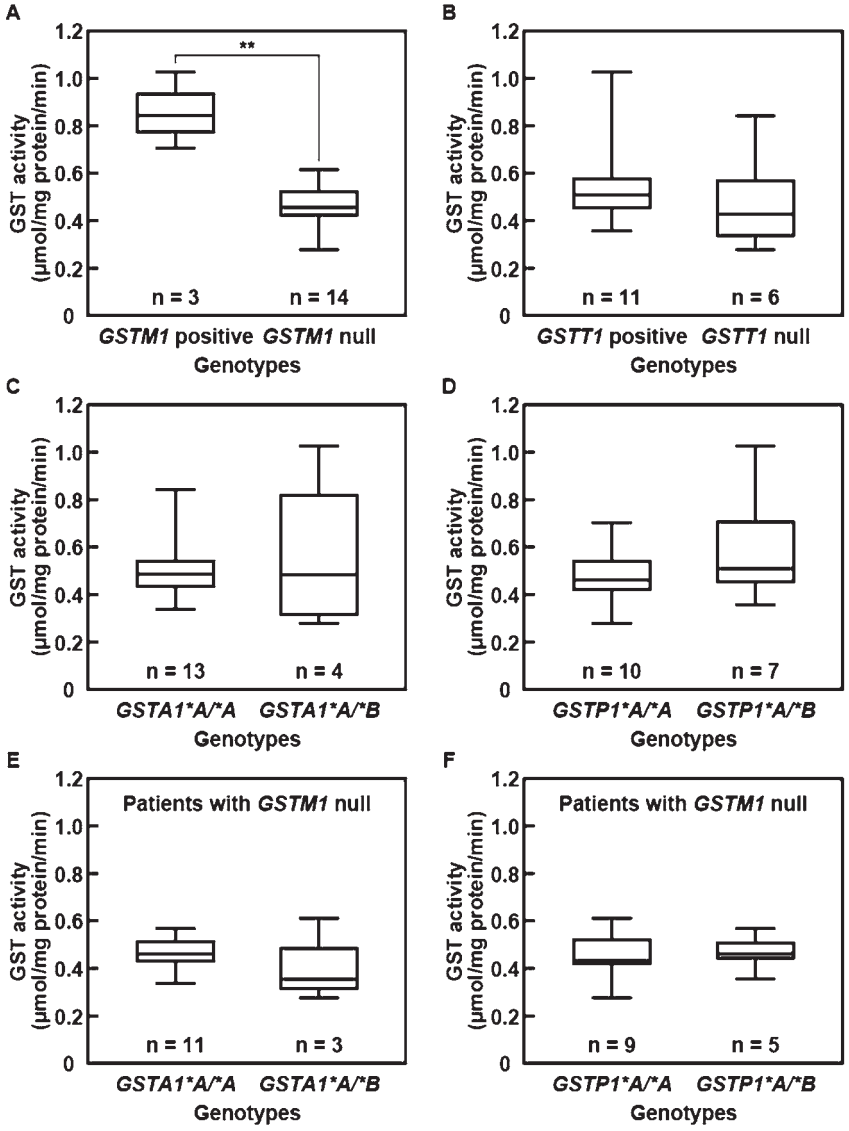

Fig. 1. Relationship between GST activity and four GST isoform genotypes (*GSTM1*, *GSTT1*, *GSTP1*, *GSTA1*; A-D) in human liver (n = 17). Panels E and F show the relationship between GST activity and *GSTA1* (E, $^*A/^*A$ and $^*A/^*B$) or *GSTP1* (F, $^*A/^*A$ and $^*A/^*B$) genotypes carried with *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[19, 20]. 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[9].

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[10]. Their results suggest that *GSTA1* and *GSTP1* may affect GST activity. We investigated and compared GST activity between *GST* genotypes including *GSTA1*B* and *GSTP1*B* because CDNB is a general substrate used to detect the total catalytic activity of GSTM1, GSTA1 and GSTP1[21]. 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[22]. 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[10]. 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[7, 23, 24]. The aryl hydrocarbon receptor (AhR) regulates the expression of *GSTA1* and *GSTA2* mRNAs[25, 26], 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[22, 27]. 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^{105} variant allele[28]. Therefore, it is apparent that *GSTP1* genotypes affect GST activity. Although it has been reported that the *GSTA1*B* as well as the *GSTP1*B* genotypes could significantly affect GST activity[8, 24], 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

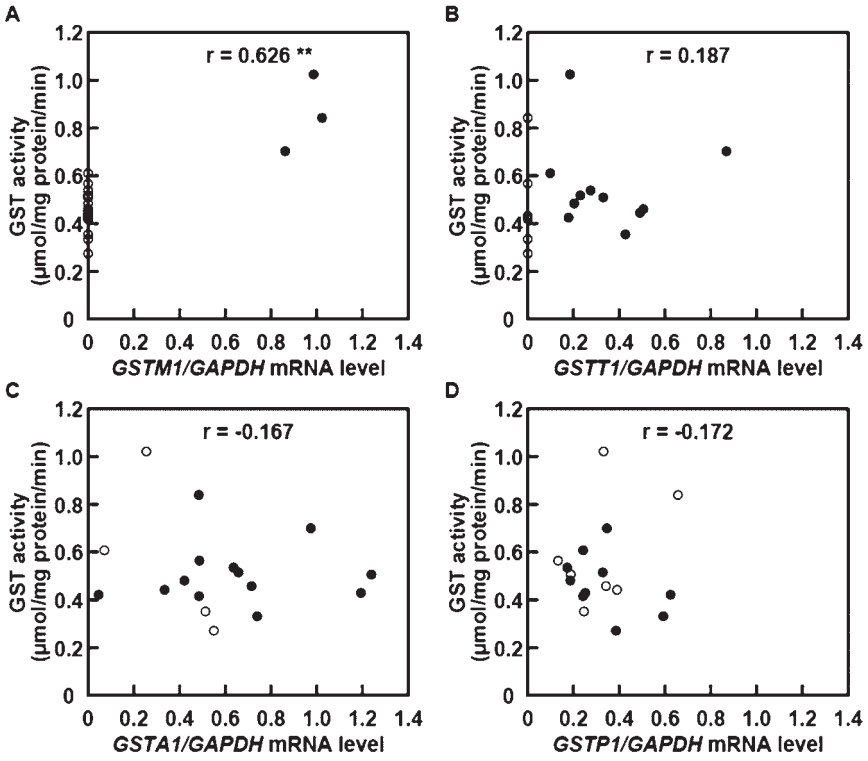

Fig. 2. The correlation between the expression of *GST* mRNA level and GST activity (n = 17). The mRNA was normalized using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as an internal control. The expression of mRNA (n = 18) in *GSTM1* positive, *GSTM1* null, *GSTT1* positive, *GSTT1* null, *GSTA1*A/*A*, *GSTA1*A/*B*, *GSTP1*A/*A* and *GSTP1*A/*B* was 0.970 ± 0.073, $4.91 \times 10^{-6} \pm 1.84 \times 10^{-5}$, 0.355 ± 0.212, $2.89 \times 10^{-4} \pm 5.98 \times 10^{-4}$, 0.645 ± 0.323, 0.348 ± 0.226, 0.400 ± 0.256 and 0.327 ± 0.171, respectively. Closed circle, wild-type; open circle, null or variant. The correlations were analyzed by the Spearman's rank correlation coefficient ($^{**}P < 0.01$). The correlation between *GSTM1* mRNA level and GST activity was significant ($P = 0.007$). No statistically significant difference was observed in *GSTT1*, *GSTA1* and *GSTP1* mRNAs with GST activity. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; r, rank correlation coefficient.

is a common substrate for measurement and evaluation of the enzymatic activities of GSTM1, GSTA1 and GSTP1 in human liver[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.

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Conflict of interest disclosure

The authors have declared no conflict of interest.

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