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Evaluation of Hepatic Glutathione S-Transferase Mu 1 and Theta 1 Activities in Humans and Mice Using Genotype Information

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GST-NULL GENOTYPES AND HEPATIC ACTIVITIES IN HUMANS AND MICE

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Abbreviations

GST, glutathione *S*-transferase; GSTM1, glutathione *S*-transferase Mu 1; GSTT1, glutathione

S-transferase Theta 1; GSH, glutathione; NBC, *p*-nitrobenzyl chloride; DCM, dichloromethane; CDNB, 1-chloro-2,4,-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; PBO, trans-4-phenyl-3-buten-2-one; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; PCR, polymerase chain reaction.

Abstract

We investigated the impact of *glutathione S-transferases Mu 1* (*GSTM1*)- and *Theta 1* (*GSTT1*)-null genotypes on hepatic GST activities in humans, and compared the results with those of *Gstm1*- and *Gstt1*-null mice. In liver with *GSTM1*/*Gstm1*-null genotype, GST activity toward *p*-nitrobenzyl chloride (NBC) was significantly decreased in both humans and mice. Additionally, in liver with *GSTT1*/*Gstt1*-null genotype, GST activity toward dichloromethane (DCM) was significantly decreased in both humans and mice. Therefore, null genotypes of *GSTM1*/*Gstm1* and *GSTT1*/*Gstt1* are considered to decrease hepatic GST activities toward NBC and DCM, respectively, in both humans and mice. This observation shows the functional similarity of GSTM1 and GSTT1 toward some substrates between humans and mice. In the case of NBC and DCM, *Gst*-null mice would be relevant models for humans with *GST*-null genotype. In addition, decreases in GST activities toward 1,2-dichloro-4-nitrobenzene, trans-4-phenyl-3-buten-2-one, and 1-chloro-2,4,-dinitrobenzene were observed in *Gstm1*-null mice, and a decrease in GST activity toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane was observed in *Gstt1*-null mice. However, an impact of *GST*-null genotypes on GST activities toward these substrates was not observed in humans. In the case of these mouse-specific substrates, *Gst*-null mice may be relevant models for humans regardless of *GST* genotype, since GST activities, which is higher in wild-type mice than in humans, were eliminated in *Gst*-null mice. This study shows that comparison of hepatic GST activities between humans and mice using genotype information would be valuable in utilization of *Gst*-null mice as human models.

Introduction

Genetic polymorphisms of the drug-metabolizing enzymes are considered significant factors that influence the incidence of toxicity by xenobiotics (Andrade et al., 2009). Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are recognized as important phase II drug-metabolizing enzymes, because they catalyze the conjugation of electrophilic compounds to glutathione (GSH), which is generally considered a detoxification reaction. Acetaminophen (Larson, 2007), bromobenzene (Lau et al., 1980), and aflatoxin B1 (Guengerich et al., 1998) are typical compounds whose reactive metabolites are detoxified by GSTs. However, in some instances, GSTs mediate metabolic bioactivation of haloalkanes such as dichloromethane (DCM) and dibromoethane (van Bladeren, 2000). Human *GSTs* display genetic polymorphisms (Hayes et al., 2005), and they have been considered significant factors that affect inter-individual differences in response to xenobiotics. Especially, null genotypes of *GSTM1* (Seidegard et al., 1988) and *GSTT1* (Pemble et al., 1994), which lack the whole gene due to homologous recombination, have been noted among *GST* polymorphisms. Furthermore, they have been reported to have significant impact on the incidence of cancer (Parl, 2005), alcoholic liver disease (Ladero et al., 2005), and drug-induced liver injury (DILI) (Lucena et al., 2008).

Metabolism catalyzed by GSTs is generally considered a detoxification reaction, which protects from DILI and xenobiotics-induced cancer (Lucena et al., 2008). Regarding DILI, the double null genotype lacking both *GSTM1* and *GSTT1* has been implicated as a risk factor for DILI induced by troglitazone

(Watanabe et al., 2003), tacrine (Simon et al., 2000), and valproic-acid (Fukushima et al., 2008). In addition, the null genotype of *GSTM1* has been suggested to be a risk factor for DILI induced by carbamazepine (Ueda et al., 2007) and antituberculosis drugs (Roy et al., 2001). One of the mechanisms of such DILI in individuals with *GSTM1*- and *GSTT1*-null genotypes is thought to be the lack of GST activity to conjugate reactive metabolites with GSH (Lucena et al., 2008). However, the direct relationship between *GST*-null genotypes and hepatic enzyme activities in humans has not been fully examined so far. While there were limited reports that focused on GST activity toward aflatoxin B1 in human liver during the 1990s (Kirby et al., 1993; Slone et al., 1995), the purpose of those studies was to examine the expression of GSTs in paired neoplastic and adjacent non-neoplastic liver tissue (Kirby et al., 1993) and to determine the extent of variation in GST activity toward aflatoxin B1-8,9-epoxide, which is a reactive metabolite of aflatoxin B1 (Slone et al., 1995). As results, decreases in GST Alpha and Mu and an increase in GST Pi were observed in neoplastic lesion, and GST activity toward aflatoxin B1-8,9-epoxide was low and showed large inter-individual variations. In addition, the impact of *GSTM1*- and *GSTT1*-null genotypes was not sufficiently examined due to the lack of information and methodology to detect *GSTM1*- and *GSTT1*-null genotypes at that time. Therefore, it seems valuable to use the information about the genotypes of *GSTM1* and *GSTT1* to examine GST activities in the liver.

As experimental animal models, several lines of *Gst*-null (knockout) mice have been produced and utilized to examine the role of *Gsts* in vivo. Regarding cytosolic GSTs, *Gsta3*- (Ilic et al., 2010), *Gsta4*-

(Engle et al., 2004), *Gstm1*- (Fujimoto et al., 2006), *Gstp1*/*p2*- (Henderson et al., 1998), *Gstt1*- (Fujimoto et al., 2007), *Gstz1*- (Fernandez-Canon et al., 2002; Lim et al., 2004), *Gsto1*- (Chowdhury et al., 2006), and *Gsts1*- (Trivedi et al., 2006) null mice have been generated. Among these *Gst*-null mice, *Gstm1*- and *Gstt1*-null mice have been developed in our group, since null genotypes of human *GSTs* have been reported to occur exclusively in *GSTM1* and *GSTT1* (Hayes et al., 2005). In order to investigate the possibility that *Gstm1*- and *Gstt1*-null mice are relevant models for humans with *GSTM1*- and *GSTT1*-null genotypes, it seems useful to examine the effect of *GSTM1*/*Gstm1*- and *GSTT1*/*Gstt1*-null genotypes on hepatic GST activities toward some specific substrates in both humans and mice. In this study, we investigated the impact of *GSTM1*- and *GSTT1*- null genotypes on hepatic enzyme activities in humans, and compared the results with those from *Gstm1*- and *Gstt1*-null mice.

Materials and Methods

Human Liver Samples

Liver samples were obtained from forty Caucasian patients who had partial hepatectomy performed due to tumor metastasis to the liver. Normal portions of the liver that had been removed together with the tumor were used for analysis. A summary of donor information is shown in Supplemental Table S1. The patients were 27 males and 13 females, and their average age ± S.D. was 62.0 ± 16.0 years. Neither human immunodeficiency virus nor hepatitis viruses B or C were detected in any sample. The obtained samples were stored in a freezer set at -80°C. The studies were approved by the Ethics Review Committee of Daiichi-Sankyo Co., Ltd., and conducted in accordance with the "Declaration of Helsinki" (1964 and subsequent revisions).

Preparation of DNA from Human Liver

DNA was extracted from liver using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) in accordance with the product instructions.

Analysis of Human *GSTM1* and *GSTT1* Genotypes

Genotypes of human *GSTM1* and *GSTT1* were determined using TaqMan® Copy Number Assays (Applied Biosystems, Foster City, CA) according to the Quick Reference Card; TaqMan® Copy Number Assays. Briefly, the assay was performed using the 7900-HT real-time polymerase chain reaction (PCR) system with a 20 uL reaction volume and using a 96 well plate containing 20 ng (4 uL) genomic DNA, 10

uL of TaqMan Gene expression Master Mix, 1 uL of TaqMan® Copy Number Assay (Hs02575461_cn or Hs00010004_cn), 1 uL of TaqMan® Copy number Reference Assays RNaseP, and 4 uL of deionized-distilled water. Quantitative PCR was conducted using the following cycling conditions: absolute quantification, 95°C for 10 min hold and 40 cycles of 95°C for 15 s and 60°C for 1 min. Each individual sample was analyzed in quadruplicate, and at least one calibrator sample for *GSTM1* and *GSTT1* (NA17122: Coriell Institute for Medical Research, Camden, NJ) was included. Copy number of *GSTM1* and *GSTT1* in the calibrator sample was 2 copies according to the product instruction. Obtained data were analyzed by CopyCaller™ Software v1.0 (Applied Biosystems) according to the product instruction.

***Gstm1*- and *Gstt1*-Null Mice**

Gstm1- and *Gstt1*- null mice were generated by homologous recombination in embryonic stem cells as described previously (Fujimoto et al., 2006; Fujimoto et al., 2007). Wild-type, *Gstm1*-null, and *Gstt1*- null mice were maintained in a C57BL/6J and 129S1 mixed background. Liver was collected from wild-type, heterozygotes, and homozygotes mice at 7 to 8 weeks of age, and the obtained liver samples were stored in a freezer set at -80°C. The studies were approved by the Ethics Review Committee for Animal Experimentation of Daiichi-Sankyo Co., Ltd., and conducted in compliance with the “Law Concerning the Protection and Control of Animals”, Japanese Law No. 105, October 1, 1973, revised on June 22, 2005.

Preparation of Cytosol from Human and Mouse Liver

Frozen liver samples were thawed and homogenized with 1.15% potassium chloride (1:3, w/v) in an ice bath. The homogenates were centrifuged at 9,000g for 20 min at 4°C, and the supernatant fractions were further centrifuged at 105,000g for 1 h at 4°C to isolate the cytosolic fraction. Protein concentrations in the cytosolic fractions were determined by the method of Lowry et al. (Lowry et al., 1951).

Western Blot Analysis of GSTM1 and GSTT1

For the Western blot analysis, the protein concentration of the cytosol was adjusted to 6 mg/mL with 1.15% potassium chloride and subsequently diluted to 3 mg/mL with Tris-SDS beta-mercaptoethanol sample loading buffer (Cosmo Bio Co., Ltd., Tokyo, Japan). Then samples were heated at 95°C for 5 minutes, and 10 µL (30 µg) of each sample was loaded onto 12.5% polyacrylamide gel (Funakoshi Corporation, Tokyo, Japan) and subjected to electrophoresis. As positive controls, 1, 5, 10, and 100 ng of recombinant human GSTM1 and GSTT1 proteins (Oxford Biomedical Research, Inc., Rochester, MI) were loaded in each gel. The proteins were transferred from the gel to an Immobilon PVDF membrane (Millipore Corporation, Billerica, MA) using a blotting apparatus (Horizeblot: Atto Corporation, Tokyo, Japan). This membrane was blocked with ECL blocking agent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and treated successively with primary antibodies. Anti-human GSTM1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-human GSTT1 antibody (Proteintech Group, Inc., Chicago, IL) were used as primary antibodies. Then, the membrane was treated with biotin-labeled

anti-rabbit IgG antibody (GE Healthcare) as the secondary antibody and finally treated with streptavidin-horseradish peroxidase conjugate (GE Healthcare). Protein-antibody complexes were detected using ECL Western blotting detection reagent (GE Healthcare) and the membrane was exposed to instant film (Fujifilm Corporation, Tokyo, Japan). Intensity of the protein bands was quantified with CS Analyzer (Atto Corporation).

Measurement of GST Activities in Human and Mouse Liver

GST activities were spectrophotometrically measured using 1-chloro-2,4,-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), *p*-nitrobenzyl chloride (NBC), trans-4-phenyl-3-buten-2-one (PBO), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), and dichloromethane (DCM) as substrates for GSTs. Since many studies using these substrates have characterized the enzymatic properties of GST isoforms (Hayes and Pulford, 1995), we also used them in this study. CDNB was used as a general substrate to detect total GST activity, which is catalyzed by various GST isoforms. NBC, DCNB, and PBO were used as substrates for Mu class GSTs, and EPNP and DCM were used as substrates for Theta class GSTs. GST activities toward CDNB (GST-CDNB activity), DCNB (GST-DCNB activity), NBC (GST-NBC activity), PBO (GST-PBO activity), and EPNP (GST-EPNP activity) were measured according to the method of Habig et al (Habig et al., 1974). GST activity toward DCM (GST-DCM activity) was measured according to the method of Nash (Nash, 1953).

Statistical Analyses

In the study using human liver samples, nonparametric statistical tests were applied for evaluating the effect of GST genotypes on hepatic GST activities or protein expression levels, since GST activities and protein expression levels did not show normal distribution as evaluated by Shapiro-Wilk test (data not shown). Human data were not analyzed by gender, since gender differences in GST activities and protein expression levels were not detected by Kruskal-Wallis test (data not shown). The association between genotype and hepatic GST activities or protein expression levels was evaluated by Jonckheere-Terpstra trend test or Kruskal-Wallis test under the assumption of the trend or genotype mode in the laws of inheritance, respectively. Correlation between protein expression levels and GST activities was analyzed by Spearman rank correlation test. Statistical software R version 2.11.0 (http://www.r-project.org) was used for all statistical analyses of data from human samples. In the study using *Gst*-null mice, the effect of *Gst* genotypes on hepatic GST activities was analyzed by a parametric Dunnett's test in accordance with the previous reports (Fujimoto et al., 2006; Fujimoto et al., 2007). Mouse data were analyzed by gender, since gender differences in GST activities toward CDNB, DCNB, NBC, and DCM were detected by F-*t* test (data not shown). Statistical analyses in the study using *Gst*-null mice were conducted with statistical software (SAS System version 6.1.2: SAS Institute Inc., Cary, NC, USA). A 5% level of probability was considered to be statistically significant in all statistical analyses performed in this study.

Results

Analysis of Human *GSTM1* and *GSTT1* Genotypes

The results of human *GSTM1* and *GSTT1* genotyping are shown in Table 1. Regarding *GSTM1*, the number of samples that had 0 copies (null genotype or homozygotes), 1 copy (heterozygotes), and 2 copies (wild-type) were 22, 15, and 3, respectively. Thus, the null allele frequency of *GSTM1* was 73.8 %. Regarding *GSTT1*, the number of samples that had 0 copies (null genotype or homozygotes), 1 copy (heterozygotes), and 2 copies (wild-type) were 7, 18, and 15, respectively. Thus, the null allele frequency of *GSTT1* was 40.0 %. The results of the combination analysis for *GSTM1* and *GSTT1* genotypes are shown in Supplemental Table S2. Four samples (10 %) out of a total of 40 samples had the *GSTM1*/*GSTT1*- double null genotype.

Western Blot Analysis of GSTM1 and GSTT1 in Human Liver

GSTM1 and GSTT1 protein expression was not detected in the human liver samples with *GSTM1*- and *GSTT1*-null genotype, respectively (Supplemental Fig. S1 and S2). Protein expression levels of GSTM1 and GSTT1 significantly changed with the copy number of *GSTM1* and *GSTT1*, respectively (Fig. 1A and 1B).

Measurement of GST activities in the Human and Mouse Liver

GST-CDNB activity, which is an indicator of total GST activity, did not significantly change with the copy number of *GSTM1* and *GSTT1* in human liver (Fig. 2A and 2B). In mouse liver, we have reported

that GST-CDNB activity significantly decreased in both *Gstm1*-heterozygotes and *Gstm1*-homozygotes (*Gstm1*-null mice) (Fujimoto et al., 2006) (Fig. 3A), but neither in *Gstt1*-heterozygotes nor *Gstt1*-homozygotes (*Gstt1*-null mice) (Fujimoto et al., 2007) (Fig 3B). Regarding Mu class substrates, GST-NBC activity significantly changed with the copy number of *GSTM1* in human liver (Fig. 4A). In contrast, GST-DCNB activity did not significantly change with the copy number of *GSTM1* in human liver (Fig. 4B). GST-DCNB activity was close to the lower limit of quantification, and GST-PBO activity was below the lower limit of quantification in human liver (data not shown). In addition, expression levels of GSTM1 protein and GST-NBC activity were significantly correlated in human liver (Fig. 4C). In mouse liver, GST-NBC activity significantly decreased in both *Gstm1*-heterozygotes and *Gstm1*-homozygotes (*Gstm1*-null mice) (Fig. 5A). We have reported that GST-DCNB activity significantly decreased in both *Gstm1*-heterozygotes and *Gstm1*-homozygotes (*Gstm1*-null mice) (Fujimoto et al., 2006) (Fig. 5B). Furthermore, GST-PBO activity significantly decreased in *Gstm1*-homozygotes (*Gstm1*-null mice) (Fig. 5C). Regarding Theta class substrates, GST-DCM activity significantly changed with the copy number of *GSTT1* in human liver (Fig. 6A). In addition, protein expression levels of GSTT1 and GST-DCM activity significantly correlated (Fig. 6B) and GST-EPNP activity was below of the lower limit of quantification (data not shown). In mouse liver, we have reported that GST-DCM and GST-EPNP activities significantly decreased in both *Gstt1*-heterozygotes and *Gstt1*-homozygotes (*Gstt1*-null mice) (Fujimoto et al., 2007) (Fig. 7A and 7B).

Discussion

We investigated the impact of *GSTM1*- and *GSTT1*-null genotypes on hepatic GST activities in humans, and compared the results with those of *Gstm1*- and *Gstt1*-null mice to evaluate the possibility that these mice are human relevant models. The null allele frequency of *GSTM1* and *GSTT1* in the human samples used in this study was 73.8 and 40.0 %, respectively (Table 1), and similar to the previously reported null allele frequency (Moyer et al., 2007), suggesting the samples used in this study were within the general distribution range of *GSTM1*- and *GSTT1*-null alleles. Since protein expression of GSTM1 and GSTT1 was not detected in the human liver samples with the *GSTM1* and *GSTT1*-null genotypes, respectively (Supplemental Fig. S1 and S2), it is suggested that the *GSTM1* and *GSTT1*-null genotypes were consistent with the absence of protein expression. In addition, protein expression levels of GSTM1 and GSTT1 significantly changed with the copy number of *GSTM1* and *GSTT1*, respectively (Fig. 1A and 1B). This result was in accord with the report that investigated the impact of *GSTM1*- and *GSTT1*-null genotypes on gene expression levels in lymphoblastoid cell lines from humans (McCarroll et al., 2006).

Among the hepatic GST activities investigated in this study using various substrates, GST-NBC and GST-DCM activities significantly decreased in liver with the *GSTM1*/*Gstm1*- and *GSTT1*/*Gstt1*-null genotypes, respectively, in both humans and mice (Fig. 4A, 5A, 6A, and 7A). Therefore, it is suggested that the impact of *GSTM1*/*Gstm1* and *GSTT1*/*Gstt1*-null genotypes was reflected in decreases in GST-NBC and GST-DCM activities, respectively, in both humans and mice. Significant correlation

between protein expression levels and activities in human liver (Fig. 4C and 6B) indicates that NBC and DCM are substrates for human GSTM1 and GSTT1, respectively. NBC has been reported as a substrate for GSTM1 in humans (Hayes and Pulford, 1995), although there has been no report about mice. Accordingly, this study shows that NBC is a substrate for GSTM1 in mice as well as in humans. DCM is a widely used industrial organic solvent, and has been reported to be a substrate for GSTT1 in both humans and mice based on studies using recombinant GSTT1 protein (Sherratt et al., 2002). However, the impact of *GSTT1*-null genotype on GST-DCM activity in human liver has not been reported so far, although we have reported that GST-DCM activity was almost absent in liver of *Gstt1*-null mice (Fujimoto et al., 2007). Therefore, this study shows that the decrease in GST-DCM activity in human liver with *GSTT1*-null genotype. From the results of the GST-NBC and GST-DCM activities, functional similarity of GSTM1 and GSTT1 between humans and mice was suggested in this study. In the case of common substrates between humans and mice, such as NBC and DCM, *Gstm1*- and *Gstt1*-null mice are considered to be relevant models of humans with *GSTM1*- and *GSTT1*-null genotypes, respectively.

Regarding DCNB, PBO, and EPNP, decreases in GST activity toward these substrates were observed specifically in *Gstm1*-null or *Gstt1*-null mice (Fig. 5B, 5C, and 7B), while an impact of *GSTM1* or *GSTT1*-null genotypes was not observed in humans (Fig. 4B). In humans, GST-DCNB activity was close to the lower limit of quantification, and GST-PBO and GST-EPNP activities were lower limit of quantification. In wild-type mice, in contrast, GST-DCNB, GST-PBO, and GST-EPNP activities were

clearly detected. Therefore, the higher GST activities toward these substrates in wild-type mice than those in humans might mask the toxicity that could occur in humans. In the case of these mouse-specific substrates, *Gst*-null mice may be relevant models for humans regardless of *GST* genotype, since GST activities, which is higher in wild-type mice than in humans, were eliminated in *Gst*-null mice. As an example of the utilization of *Gst*-null mice, it has been reported that *Gsta3*-null mice are sensitive to aflatoxin B1-induced cytotoxicity and genotoxicity (Ilic et al., 2010). This fact suggests the usefulness of *Gsta3*-null mice as an in vivo model to assess the risk of aflatoxin B1 in humans, since wild-type mice are resistant to the aflatoxin B1-induced toxicity due to high GST activity toward aflatoxin B1, which is mainly catalyzed by GSTA3. In addition, *Gstm1*-null mice showed marked methemoglobinemia in a single dose study of DCNB compared with wild-type mice (Arakawa et al., 2010). GST-DCNB activity in both humans and *Gstm1*-null mice was close to the lower limit of quantification, and the absolute value of GST-DCNB activity was similar between humans (2.5 ± 1.3 nmol/min/mg protein) and *Gstm1*-null mice (Male: 5.1 ± 1.9 nmol/min/mg protein, Female: 3.2 ± 2.0 nmol/min/mg protein). Considering the absolute value of GST-DCNB activity, the results from *Gstm1*-null mice may be appropriate for assessment of the human risks to DCNB. In other words, the higher GST-DCNB activity in wild-type mice than in humans might mask the toxicity that could occur in humans. To avoid underestimating human risk from exposure to DCNB, the marked methemoglobinemia induced by single doses of DCNB in *Gstm1*-null mice should be considered to indicate a potential risk in humans.

Regarding CDNB, which is an general substrate for various GSTs, a decrease in GST-CDNB activity was observed in *Gstm1*-null mice (Fig. 3A), but not in humans with *GSTM1*-null genotype (Fig. 2A). Since it has been reported that GSTM1 of mice and humans shows similar activity toward CDNB (Hayes and Pulford, 1995), GSTM1 expression levels among total GSTs in liver might be higher in mice than in humans. No decrease in GST-CDNB activity was observed in either *Gstt1*-null mice (Fig. 3B) or humans with *GSTT1*-null genotype (Fig. 2B). These results were consistent with the report showing that GSTT1 lacks activity toward CDNB in both mice (Whittington et al., 1999) and humans (Sherratt et al., 1997).

In drug development, it is important to consider and predict the effect of genetic polymorphisms on the efficacy and safety of candidate compounds (Ma and Lu, 2011). In addition, a screening system for reactive metabolites to reduce idiosyncratic DILI recently become important in the absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) studies (Ikeda, 2011). *Gstm1*- and *Gstt1*-null mice might be important in vivo models to evaluate the formation of reactive metabolite or the subsequent covalent binding and toxicity in ADME/Tox studies. Further efforts to utilize *Gstm1*- and *Gstt1*-null mice will be necessary, since experimental data of these models are limited so far.

The impact of *GSTM1*/*Gstm1* and *GSTT1*/*Gstt1* on hepatic GST activities is summarized in Table 2 and Table 3, respectively. In conclusion, comparison of GST activities between humans and mice in liver with genotype information would be valuable in utilization of *Gst*-null mice as human models.

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Authorship Contributions

Participated in research design: Arakawa, Shinagawa, Fischer, Mueller, and Takasaki.

Conducted experiments: Arakawa, Fujimoto, Kato, Endo, Fukahori, and Fischer.

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Wrote or contributed to the writing of the manuscript: Arakawa and Fujimoto.

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Footnotes

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Legends for Figures

Fig. 1. Relationship between *GST* copy number and protein expression levels in human liver. A, Relationship between *GSTM1* copy number and protein expression levels of GSTM1. B, Relationship between *GSTT1* copy number and protein expression levels of GSTT1. Data are shown as individual points, and filled circles and open triangles indicate males and females, respectively. *GSTM1* and *GSTT1* copy numbers were determined by TaqMan® Copy Number Assays (Applied Biosystems), and protein expression levels of GSTM1 and GSTT1 were determined by Western blot analysis and quantification of band intensity using CS Analyzer (Atto Corporation).

Fig. 2. Impact of *GSTM1*- and *GSTT1*-null genotype on GST-CDNB activity in human liver. A, Relationship between *GSTM1* copy number and GST-CDNB activity. B, Relationship between *GSTT1* copy number and GST-CDNB activity. Data are shown as individual points, and filled circles and open triangles indicate males and females, respectively. *GSTM1* and *GSTT1* copy numbers were determined by TaqMan® Copy Number Assays (Applied Biosystems), and GST-CDNB activity was measured spectrophotometrically.

Fig. 3. Impact of *Gstm1*- and *Gstt1*-null genotype on GST-CDNB activity in mouse liver. A, Relationship between *Gstm1* genotypes and GST-CDNB activity (Data from Fujimoto et al., *Drug Metab*

Dispos 34: 1495-1501, 2006). B, Relationship between *Gstt1* genotypes and GST-CDNB activity (Data from Fujimoto et al., *Drug Metab Dispos* 35: 2196-2202, 2007). Open, light gray, and dark gray bars indicate wild-type, *Gst*-heterozygotes, and *Gst*-homozygotes (*Gst*-null mice), respectively. The values are depicted as the mean ± S.D. of four or five mice per group. GST-CDNB activity was measured spectrophotometrically. Significant differences from the wild-type by Dunnett's test are shown as ** $P < 0.01$ and *** $P < 0.001$.

Fig. 4. Impact of *GSTM1*-null genotype on GST-NBC and GST-DCNB activities in human liver. A, Relationship between *GSTM1* copy number and GST-NBC activity. B, Relationship between *GSTM1* copy number and GST-DCNB activity. C, Relationship between protein expression levels of GSTM1 and GST-NBC activity. Data are shown as individual points, and filled circles and open triangles indicate males and females, respectively. *GSTM1* copy number was determined by TaqMan® Copy Number Assays (Applied Biosystems), and GST-NBC and GST-DCNB activities were measured spectrophotometrically. Protein expression levels of GSTM1 was determined by Western blot analysis and quantification of band intensity using CS Analyzer (Atto Corporation).

Fig. 5. Impact of *Gstm1*-null genotype on GST-NBC, GST-DCNB, and GST-PBO activities in mouse liver. A, Relationship between *Gstm1* genotypes and GST-NBC activity. B, Relationship between *Gstm1*

genotypes and GST-DCNB activity (Data from Fujimoto et al., *Drug Metab Dispos* 34: 1495-1501, 2006). C, Relationship between *Gstm1* genotypes and GST-PBO activity. Open, light gray, and dark gray bars indicate wild-type, *Gstm1*-heterozygotes, and *Gstm1*-homozygotes (*Gstm1*-null mice), respectively. The values are depicted as the mean ± S.D. of four or five mice per group. GST-NBC, GST-DCNB, and GST-PBO activities were measured spectrophotometrically. Significant differences from the wild-type by Dunnett's test are shown as ** $P < 0.01$ and *** $P < 0.001$.

Fig. 6. Impact of *GSTT1*-null genotype on GST-DCM activity in human liver. A, Relationship between *GSTT1* copy number and GST-DCM activity B, Relationship between protein expression levels of GSTT1 and GST-DCM activity. Data are shown as individual points, and filled circles and open triangles indicate males and females, respectively. *GSTT1* copy number was determined by TaqMan® Copy Number Assays, and GST-DCM activity was measured spectrophotometrically. Protein expression levels of GSTT1 was determined by Western blot analysis and quantification of band intensity using CS Analyzer (Atto Corporation).

Fig. 7. Impact of *Gstt1*-null genotype on GST-DCM and GST-EPNP activities in mouse liver (Data from Fujimoto et al., *Drug Metab Dispos* 35: 2196-2202, 2007). A, Relationship between *Gstt1* genotypes and GST-DCM activity. B, Relationship between *Gstt1* genotypes and GST-EPNP activity. Open, light

gray, and dark gray bars indicate wild-type, *Gstt1*-heterozygotes, and *Gstt1*-homozygotes (*Gstt1*-null mice), respectively. GST-DCM and GST-EPNP activities were measured spectrophotometrically. Significant differences from the wild-type by Dunnett's test are shown as *** $P < 0.001$.

TABLE 1 *Analysis of Human GSTM1 and GSTT1 Genotypes*

Gene	Copy Number/ Genotype			No. Examined	Null Allele Frequency
	0 copies	1 copy	2 copies		
	Homozygotes (Null)	Heterozygotes	Wild-type		
GSTM1	22 (55.0 %)	15 (37.5 %)	3 (7.5 %)	40	73.8 %
GSTT1	7 (17.5 %)	18 (45.0 %)	15 (37.5%)	40	40.0 %

TABLE 2 *Summary of the impact of GSTM1/Gstm1-null genotype on GST activities*

Isoform Specificity	**Substrate**	**Human *GSTM1*-null**	**Mouse *Gstm1*-null**	**Substrate Classification**
Total GSTs	CDNB	→	↓*	Mouse-specific
Mu class GSTs	NBC	↓	↓	Common
	DCNB	→ (CLLOQ)	↓*	Mouse-specific
	PBO	→ (BLLOQ)	↓	Mouse-specific

→: No impact, ↓: Decreased, CLLOQ: Close to the lower limit of quantification, BLLOQ: Below the lower limit of quantification

*: Data from Fujimoto et al., *Drug Metab Dispos* 34: 1495-1501, 2006

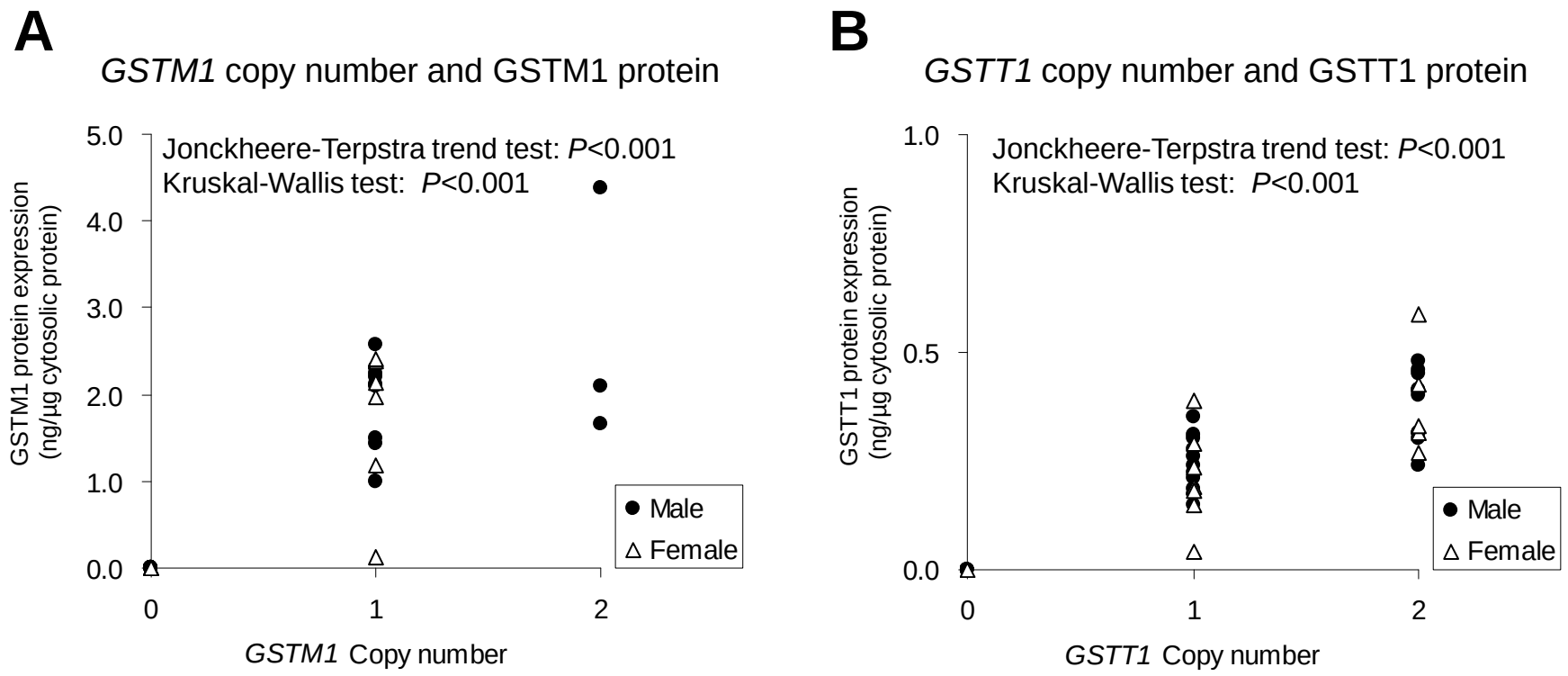
TABLE 3 *Summary of the impact of GSTT1/Gstt1-null genotype on GST activities*

Isoform Specificity	**Substrate**	**Human *GSTT1*-null**	**Mouse *Gstt1*-null**	**Substrate Classification**
Total GSTs	CDNB	→	→*	Not substrate
Theta class GSTs	DCM	↓	↓*	Common
	EPNP	→ (BLLOQ)	↓*	Mouse-specific

→: No impact, ↓: Decreased, BLLOQ: Below the lower limit of quantification

*: Data from Fujimoto et al., *Drug Metab Dispos* 35: 2196-2202, 2007

Fig. 1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GSTM1 copy number and GSTM1 protein
Jonckheere-Terpstra trend test: *P*<0.001
Kruskal-Wallis test: *P*<0.001
GSTM1 protein expression (ng/µg cytosolic protein)
5.0
4.0
3.0
2.0
1.0
0.0
0
1
2
GSTM1 Copy number
Male
Female
B
GSTT1 copy number and GSTT1 protein
Jonckheere-Terpstra trend test: *P*<0.001
Kruskal-Wallis test: *P*<0.001
GSTT1 protein expression (ng/µg cytosolic protein)
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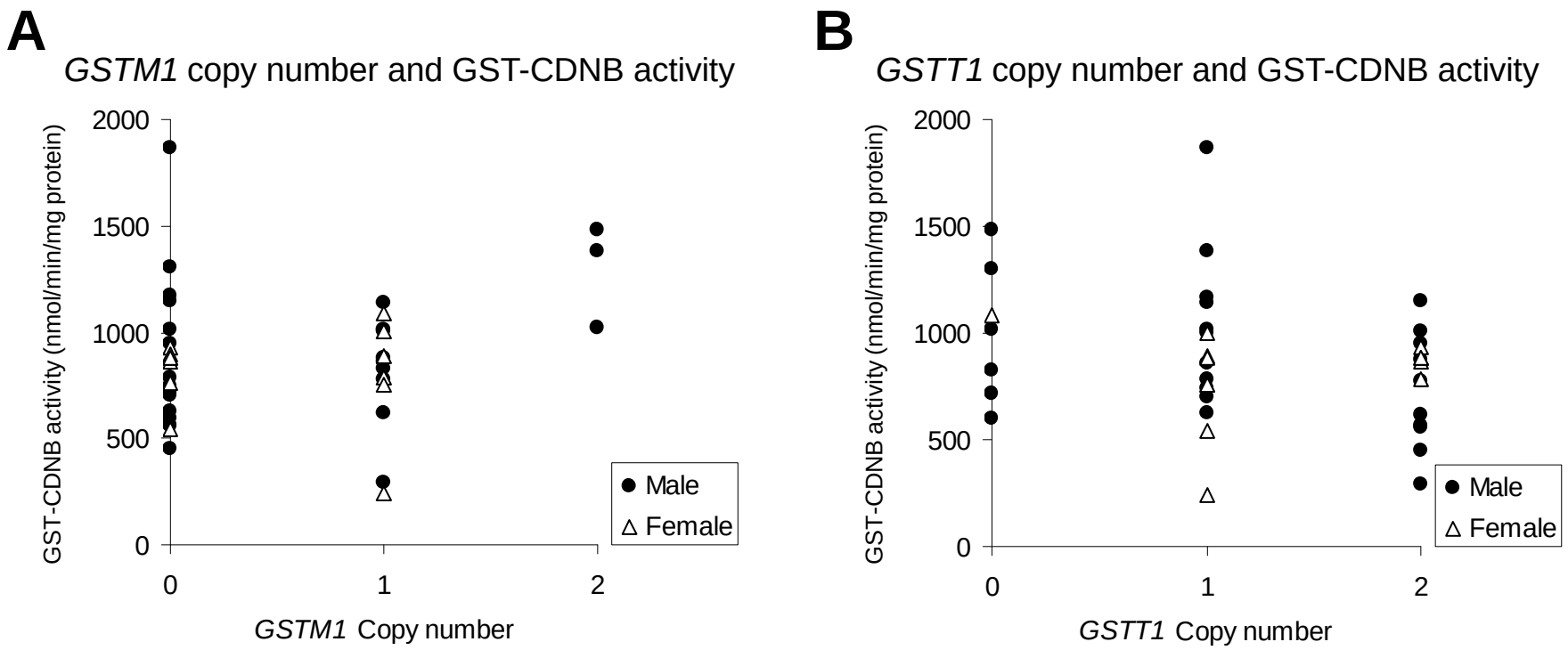
0.5
0.0
0
1
2
GSTT1 Copy number
Male
Female

Fig. 2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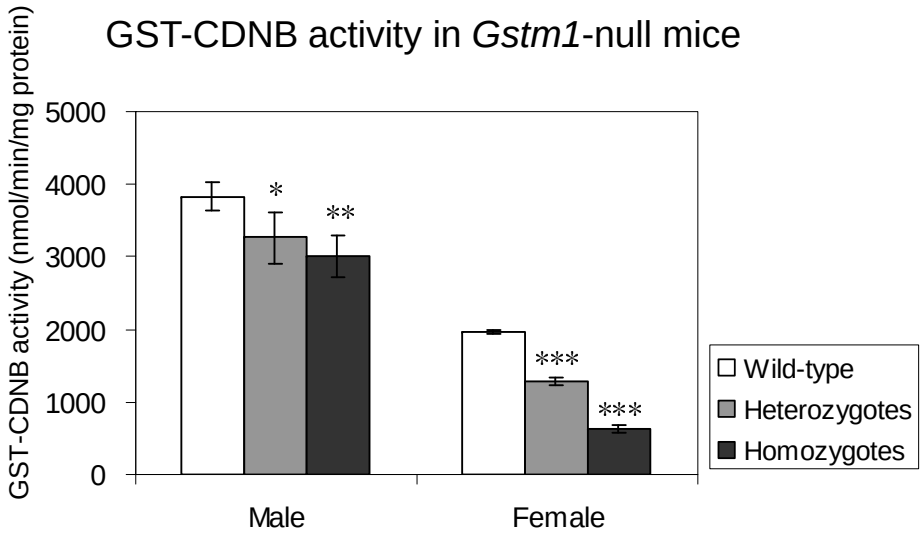
GSTM1 copy number and GST-CDNB activity
GST-CDNB activity (nmol/min/mg protein)
2000
1500
1000
500
0
0
1
2
GSTM1 Copy number
Male
Female
B
GSTT1 copy number and GST-CDNB activity
GST-CDNB activity (nmol/min/mg protein)
2000
1500
1000
500
0
0
1
2
GSTT1 Copy number
Male
Female

Fig. 3

A


GST-CDNB activity in *Gstm1*-null mice
GST-CDNB activity (nmol/min/mg protein)
5000
4000
3000
2000
1000
0
*
**
***
***
Male
Female
Wild-type
Heterozygotes
Homozygotes

B

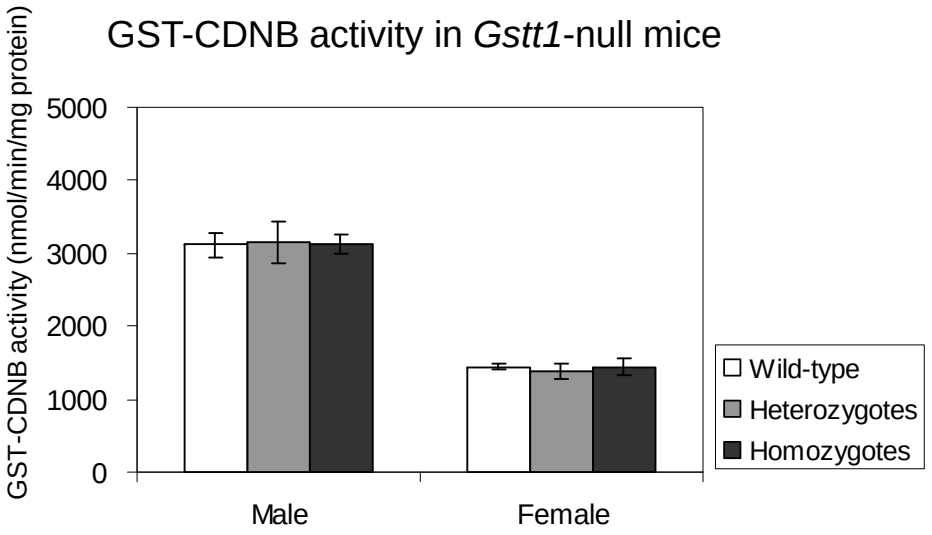

GST-CDNB activity in *Gstt1*-null mice
GST-CDNB activity (nmol/min/mg protein)
5000
4000
3000
2000
1000
0
Male
Female
Wild-type
Heterozygotes
Homozygotes

Fig. 4

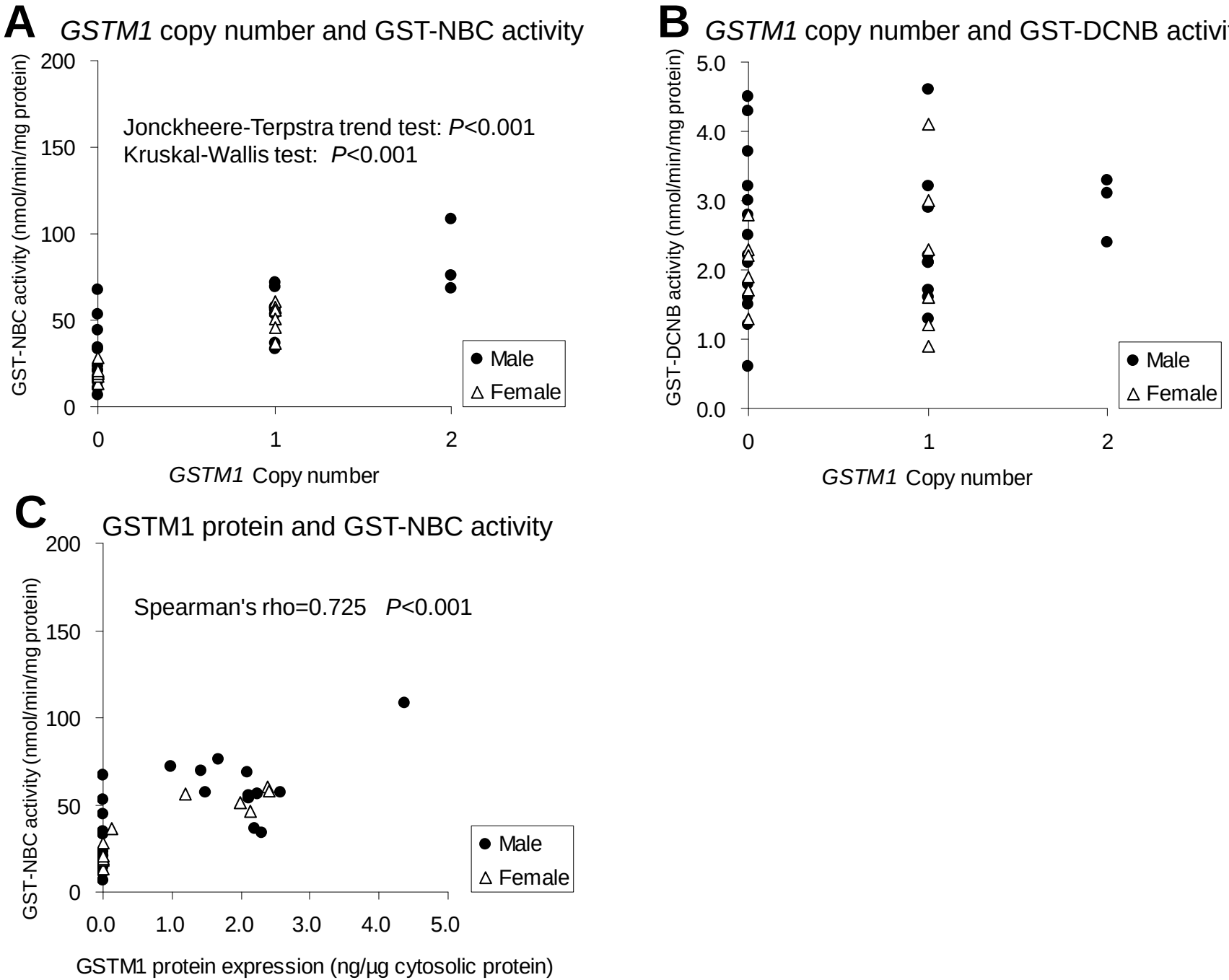

A
GSTM1 copy number and GST-NBC activity
GST-NBC activity (nmol/min/mg protein)
200
150
100
50
0
Jonckheere-Terpstra trend test: P<0.001
Kruskal-Wallis test: P<0.001
Male
Female
0
1
2
GSTM1 Copy number
B
GSTM1 copy number and GST-DCNB activity
GST-DCNB activity (nmol/min/mg protein)
5.0
4.0
3.0
2.0
1.0
0.0
Male
Female
0
1
2
GSTM1 Copy number
C
GSTM1 protein and GST-NBC activity
GST-NBC activity (nmol/min/mg protein)
200
150
100
50
0
Spearman's rho=0.725 P<0.001
Male
Female
0.0
1.0
2.0
3.0
4.0
5.0
GSTM1 protein expression (ng/µg cytosolic protein)

Fig. 5

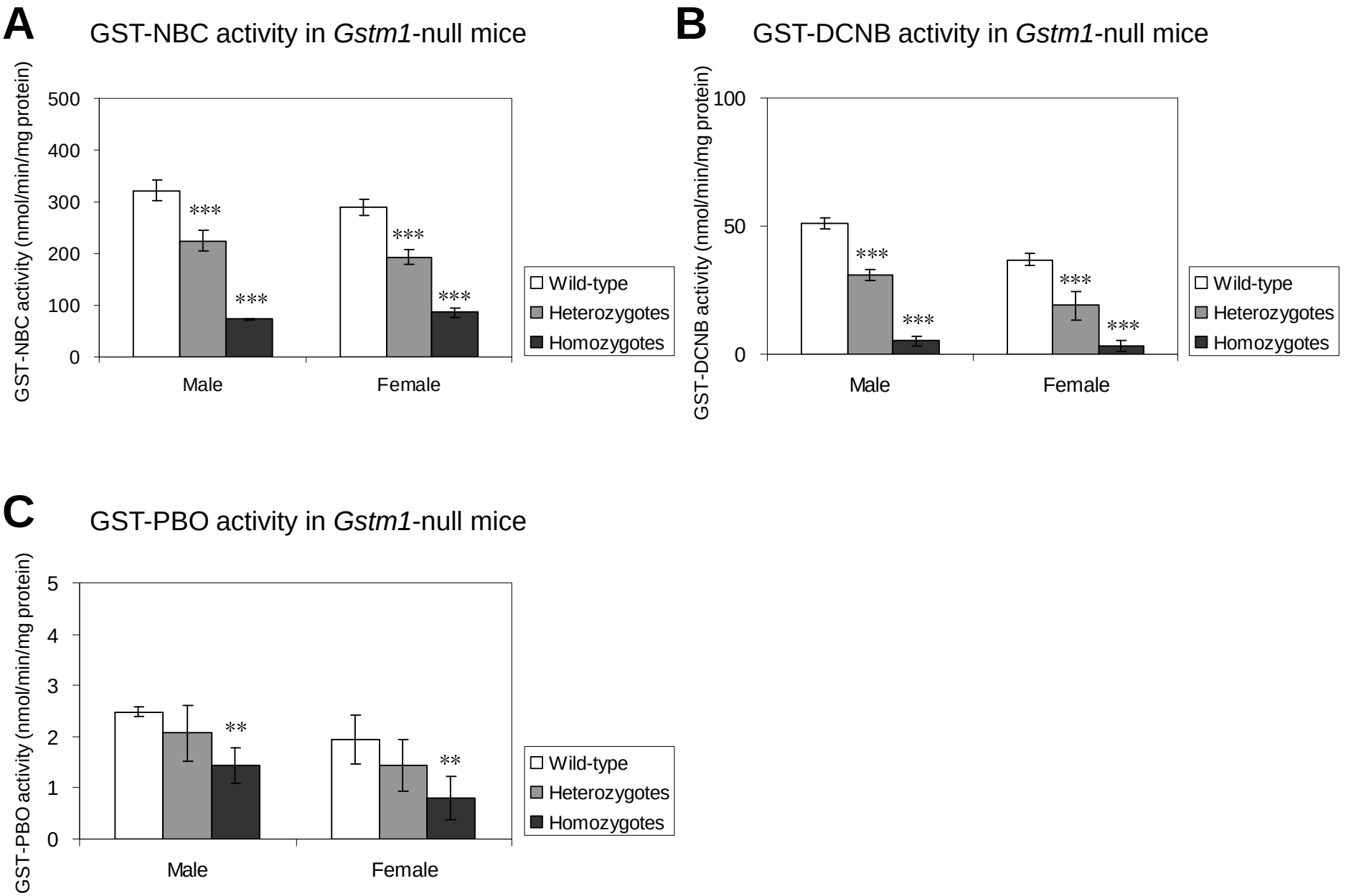

A
GST-NBC activity in *Gstm1*-null mice
GST-NBC activity (nmol/min/mg protein)
500
400
300
200
100
0
***
***
***
***
Male
Female
Wild-type
Heterozygotes
Homozygotes
B
GST-DCNB activity in *Gstm1*-null mice
GST-DCNB activity (nmol/min/mg protein)
100
50
0
***
***
***
***
Male
Female
Wild-type
Heterozygotes
Homozygotes
C
GST-PBO activity in *Gstm1*-null mice
GST-PBO activity (nmol/min/mg protein)
5
4
3
2
1
0
**
**
Male
Female
Wild-type
Heterozygotes
Homozygotes

Fig. 6

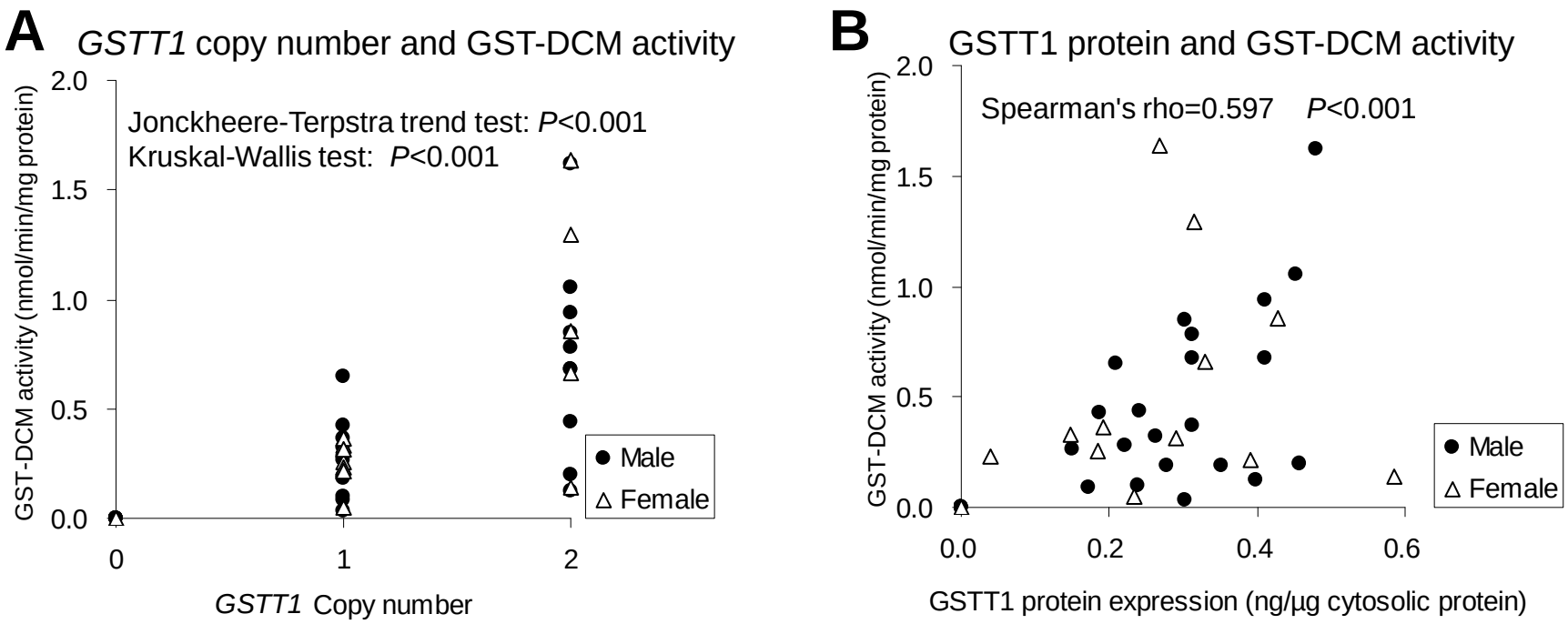
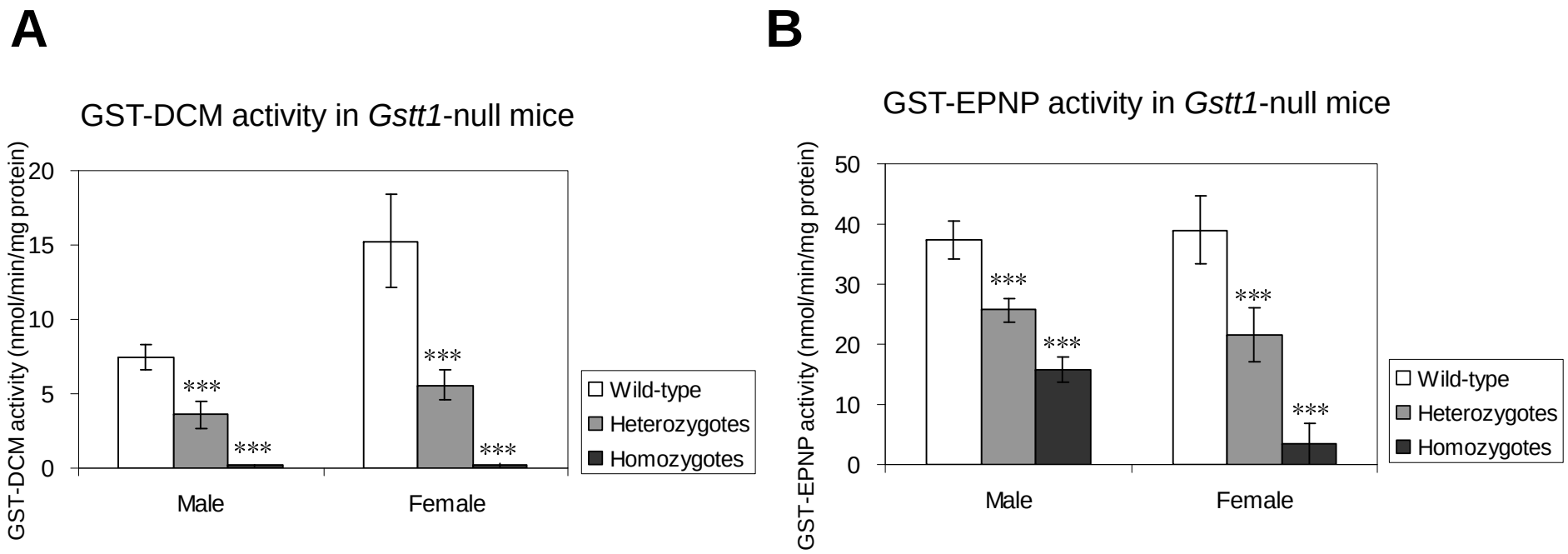

A
GSTT1 copy number and GST-DCM activity
Jonckheere-Terpstra trend test: P<0.001
Kruskal-Wallis test: P<0.001
GST-DCM activity (nmol/min/mg protein)
2.0
1.5
1.0
0.5
0.0
0
1
2
GSTT1 Copy number
Male
Female
B
GSTT1 protein and GST-DCM activity
Spearman's rho=0.597 P<0.001
GST-DCM activity (nmol/min/mg protein)
0.0
0.2
0.4
0.6
GSTT1 protein expression (ng/µg cytosolic protein)
Male
Female

Fig. 7


A
GST-DCM activity in *Gstt1*-null mice
GST-DCM activity (nmol/min/mg protein)
0
5
10
15
20
***
***
***
Male
Female
Wild-type
Heterozygotes
Homozygotes
B
GST-EPNP activity in *Gstt1*-null mice
GST-EPNP activity (nmol/min/mg protein)
0
10
20
30
40
50
***
***
***
Male
Female
Wild-type
Heterozygotes
Homozygotes